LINK TO ORIGINAL ARTICLE
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The relevance of assessment of intestinal P-gp inhibition using digoxin as an *in vivo* probe substrate

Jack G. Shi, Yan Zhang and Swamy Yeleswaram

The recent Review (Membrane transporters in drug development. *Nature Rev. Drug Discov.* **9**, 215–236; 2010)[1] by the International Transporter Consortium (ITC) proposed that, for a new molecular entity (NME) intended for oral administration, a clinical drug interaction study with digoxin should be conducted if $[I2]/IC_{50} \geq 10$ (where I2 is the hypothetical concentration of the NME in gut lumen, and IC_{50} is the NME's potency of P-glycoprotein (P-gp) inhibition *in vitro*). However, the Review does not adequately address the choice of digoxin as a suitable *in vivo* P-gp probe substrate in the context of digoxin's clinical pharmacokinetics, as well as the prevalence of drug–drug interactions due to inhibition of P-gp at the intestinal level and hence the importance of such interactions.

Digoxin is certainly a sensitive probe substrate of P-gp in *in vitro* studies, as evidenced by a high efflux ratio across Caco-2, MDR1-MDCK and MDR1-LLCPK1 cells, as well as effective inhibition of efflux in the presence of P-gp inhibitors. However, the *in vivo* situation is less clear. In humans, digoxin is subjected to minimal metabolism and its oral bioavailability approximates its fraction absorbed in the gastrointestinal tract. Administered in standard tablet formulation, digoxin exhibits good but incomplete absorption; for example, the absolute bioavailability of Lanoxin (GlaxoSmithKline) is 60–80% (REF. 2). When dosed in solubilized formulations (for example, Lanoxicaps), digoxin is almost completely absorbed[2,3], which questions the relevance of P-gp-mediated efflux in the oral absorption of digoxin. Other reports support the notion that the rate of oral absorption of digoxin is limited by dissolution (rather than permeability)[4,5]. It should also be noted that grapefruit juice, an intestinal P-gp inhibitor, has a non-significant effect on digoxin serum concentrations[6], and ketoconazole — a potent P-gp inhibitor — is not known to cause significant interaction with digoxin, which further undermines the choice of digoxin as an *in vivo* probe substrate.

Numerous reported clinical drug interactions involving digoxin and P-gp inhibitors (verapamil, clarithromycin, intraconazole, carvedilol, talinolol, nifedipine and propafenone) that are used as evidence of intestinal P-gp inhibition suffer from a critical flaw. The formulation of each of these interacting drugs contains polyethylene glycol (PEG) and/or propylene glycol (PG) as excipients. It is therefore reasonable to suspect that these interactions may have little to do with P-gp inhibition and more to do with solubilization of digoxin. It is worth noting that digoxin oral absorption is not affected when dosed in solubilized formulations, in which PEG or PG is used as a typical co-solvent[2,3,7].

Consistent with dissolution rate-limited oral absorption of digoxin, which has been well established, there is no compelling example that we could find in the literature to clearly substantiate clinical drug interaction for digoxin due to inhibition of intestinal P-gp[8]. Therefore, a digoxin-based clinical study with the objective of evaluating drug interaction effect resulting from intestinal P-gp inhibition is unlikely to produce any meaningful data. On the other hand, there is credible evidence to suggest that solubilizing or emulsifying excipients such as PEG, PG and cremophor[9] may significantly increase digoxin oral absorption and serum concentrations. Due to the narrow therapeutic index of this widely prescribed drug, we think a clinical interaction study with digoxin should be mandatory for any investigational drug product with an oral formulation containing solubility-enhancing excipients such as those discussed above, regardless of the P-gp-inhibitory potential of the active principal ingredient itself.

We recognize the need in drug development to evaluate P-gp inhibition at the gut level as a potential source of drug interaction for NMEs that are potent P-gp inhibitors *in vitro*, and suggest the following set of criteria for selecting the ideal P-gp probe for clinical evaluation of drug–drug interactions. First, an ideal probe substrate for P-gp should be a sensitive substrate of P-gp, as evidenced by its high efflux ratios in bidirectional transport assays in P-gp-expressing cell lines; second, it should be dosed low enough such that the initial concentration in the gut does not exceed its K_m for P-gp, thereby enabling P-gp-mediated efflux *in vivo*; third, the P-gp probe substrate should exhibit permeability (rather than solubility or dissolution) rate-limited oral absorption and a low-to-moderate oral bioavailability; fourth, it should be minimally metabolized (including first-pass metabolism at the gut wall), so that the pharmacokinetics is not confounded by processes affecting metabolism; fifth, it should not be a high-affinity substrate for any uptake or efflux transporter (other than P-gp) that is expressed in human intestinal enterocytes; and last, it should have adequate clinical experience from a safety standpoint. Digoxin does not meet the third requirement, and thus is not an acceptable probe drug for the purpose.

A survey of the literature indicates that molecules that possess all these qualities are rare. The β-blocker drug talinolol is a well-studied P-gp substrate with approximately tenfold efflux ratio in Caco-2 cells[10], a moderate oral bioavailability of approximately 55% for the immediate-release (IR) dosage, and negligible (<1%) metabolic clearance[11]. The limited oral bioavailability of talinolol IR tablets is unlikely to be caused by slow dissolution, as the tablet exhibits relatively rapid dissolution in aqueous media with no less than 80% of the labelled amount of the drug substance dissolved in 30 minutes at pH 6.8 and almost 100% of the drug dissolved in 10 minutes at pH 1.0 (REF. 12). With concomitant administration of erythromycin, a P-gp inhibitor, talinolol exhibited increased oral bioavailability without any change in renal clearance[13] whereas, following intestinal P-gp induction by rifampin, the oral bioavailability of talinolol was significantly decreased[14], suggesting that intestinal P-gp-mediated efflux is the rate-limiting factor for oral absorption of talinolol. Although talinolol is also a substrate of certain uptake transporters expressed in human intestines[15], its overall profile seems to be acceptable as an *in vivo* probe substrate of intestinal P-gp. Besides talinolol, there may be proprietary molecules that meet the above criteria, and we urge the ITC to encourage pharmaceutical companies to voluntarily contribute and share the data for potential model P-gp substrates in the quest to find an ideal probe P-gp substrate.

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LINK TO ORIGINAL ARTICLE
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Response from the International Transporter Consortium

Caroline A. Lee, Kathleen M. Hillgren, Lei Zhang and Joseph W. Polli, on behalf of the International Transporter Consortium

We are grateful for the comments on our article (Membrane transporters in drug development. *Nature Rev. Drug Discov.* **9**, 215–236 (2010))[1] from Shi, Zhang and Yeleswaram (The relevance of assessment of intestinal P-gp inhibition using digoxin as an *in vivo* probe substrate. 31 Dec 2010 (doi:10.1038/nrd3028-c1))[2] regarding their assessment of the utility of digoxin as an *in vivo* probe for P-glycoprotein (P-gp). Overall, the International Transporter Consortium (ITC) is in agreement with their comments and supports future research to identify better *in vivo* probes for P-gp. Below are further thoughts from the ITC on some of the salient points highlighted by Shi and colleagues.

Utility of digoxin as an* in vivo *probe. The ITC Review did highlight (in the "Decision trees for P-glycoprotein or BCRP inhibitor interactions" (Box 3)) a number of the same limitations of digoxin as a clinical probe as those pointed out by Shi and colleagues. For instance, digoxin was noted as a "unique drug" and may not be the best reference for decisions regarding interaction studies with other P-gp substrate drugs. However, owing to its narrow therapeutic index, interactions that alter the area under the curve (AUC) for digoxin ≥ 1.25-fold, whether driven by P-gp, other transporters or solubility enhancement, are clinically important for digoxin, as dose adjustment may be warranted. The ITC advised readers to be cautious and not to over-extrapolate the significance of P-gp drug–drug interactions based on the importance of these findings for digoxin.

Further guidance was also given that, if the new molecular entity (NME) is a pure P-gp inhibitor, no drug–drug interaction (DDI) studies may be required other than for clarifying a digoxin dose adjustment requirement, depending on the therapeutic area of the NME and its drug-labelling considerations. It is of crucial importance that the decision to conduct a clinical P-gp DDI study is based on an integration of all the nonclinical and clinical data for the NME. In summary, because digoxin has a narrow therapeutic index and it is a substrate for intestinal P-gp (discussed below), the ITC thinks that clinical DDI studies with drugs that are inhibitors of P-gp should be considered as described in the decision tree.

Importance of solubility and formulation on digoxin absorption. The ITC did mention the importance of considering the digoxin formulation to use in the clinical DDI study, as the different formulations of digoxin have different bioavailability values. As pointed out by the ITC and Shi and colleagues, the standard digoxin tablet has an absolute bioavailability of ~70%, whereas bioavailability for the liquid-filled formulation (that is, the Lanoxicaps capsule) is ~90%. It is of note that the Lanoxicaps capsule product has not been commercially available in the US market since March 2008 (REF. 3); the formal withdrawal due to commercial reasons was requested in July 2010.

Shi and colleagues present an interesting position that a clinical interaction study with digoxin should be mandatory for any investigational drug product with an oral formulation containing solubility-enhancing excipients, regardless of whether the investigational drug product is a P-gp inhibitor, because they hypothesize that interactions observed with digoxin may have little to do with P-gp inhibition but more to do with solubilization of digoxin by the co-formulated agents (for example, polyethylene glycol (PEG) or propylene glycol (PG)). However, Shi and colleagues did not provide detailed data in their letter to support their position. The effect of formulations on the pharmacokinetics of digoxin or other narrow therapeutic drugs was beyond the scope of the ITC report, and so the ITC does not have a formal position on this topic.

However, the US Food and Drug Administration, independent of the ITC, has been investigating the potential formulation effect by collecting information on the digoxin formulation (Lanoxin tablet versus Lanoxicaps capsule) and interacting drug formulation (PEG- or PG-containing versus non-PEG- or non-PG-containing). Their preliminary results (L. Zhang, personal communication) suggest a lack of correlation between the presence of PEG or PG in the inhibitor drug formulation and positive inhibition effect on digoxin. For example, some known non-inhibitors of digoxin, such as clopidogrel and rosiglitazone, contain PEG in their formulations, suggesting that PG or PEG in the drug formulation might not be a key factor influencing a digoxin drug interaction. The findings of this work will be reported in a future publication.

Digoxin and intestinal P-gp. Digoxin is a poor/moderately permeable P-gp substrate[4]. Shi *et al.* noted that "there is no compelling example that we could find in the literature to clearly substantiate clinical drug interaction for digoxin due to inhibition of intestinal P-gp". The ITC would like to highlight the study by Drescher *et al.*[5] in which digoxin dosed intravenously in humans was eliminated into the jejunum (measured via an intestinal catheter) and bile. In this study, digoxin intestinal elimination was reduced by approximately 50% by quinidine (a P-gp inhibitor) and increased by 100% after multiple-dose rifampicin (a P-gp inducer) treatment, demonstrating a role for intestinal P-gp[5–7]. Another indicator for intestinal P-gp interaction is based on pharmacokinetic changes in which maximum plasma concentration (C_{max}) and area under the curve (AUC) of oral digoxin increased more than intravenous digoxin, and renal clearance for oral digoxin did not change with the co-administration of amiodarone, verapamil, carvedilol or atorvastatin[8–12].

Shi *et al.* further noted that there are other P-gp inhibitor examples such as grapefruit juice and ketoconazole that do not alter the exposure of digoxin. However, although grapefruit juice did not change digoxin exposure, there was a change in the rate of absorption (k_a and t_{lag})[13]. Also the effect of grapefruit juice on intestinal P-gp will be variable among brands and is known to be dependent on the concentration and the preparation[14–16]. In the case of ketoconazole, it should be given with Coca-Cola, an acidic beverage, for complete dissolution[17,18]. Therefore, the lack of effect of ketoconazole may be attributed to poor dissolution leading to suboptimal concentrations to inhibit intestinal P-gp.

Utility of talinolol as an* in vivo *probe. Shi and colleagues, along with others, have proposed talinolol as a potential P-gp clinical probe substrate based on this drug being a good *in vitro* P-gp substrate, having an

oral bioavailability of ~55%, and negligible metabolic clearance. It also has been shown to exhibit a clinical DDI with erythromycin and rifampin[19,20]. Furthermore, the safety margin is wider for talinolol than for digoxin. These properties make talinolol a potential P-gp probe drug candidate.

However, talinolol has a number of limitations; many of these are similar to digoxin, while others unique to talinolol itself. For example, talinolol is a substrate of multiple transporters, and its pharmacokinetics from the fast-disintegrating dosage form is associated with a double absorption peak that could confound interpretation of a DDI. The pharmacokinetic interactions due to P-gp inhibition/induction seem to be fairly modest, with changes of only ~30–50% (notably lower than those with digoxin), questioning whether this change is sensitive enough to assess drug interactions; it is noteworthy that cytochrome P450 probe substrates tend to have much large changes (> 200%) when co-dosed with an inhibitor. There are also a number of other modest interactions (~20–35%) with unknown mechanisms; for example, the P-gp inhibitor verapamil reduces exposure whereas one would have predicted an increase in exposure[21]. Most importantly, talinolol is only approved in Germany, which limits the utility of the drug in other countries including the United States. These limitations do not preclude the use of talinolol as a P-gp probe, but are key considerations when designing a clinical P-gp DDI study, in particular if one considers how the information obtained with talinolol can be extrapolated to other P-gp substrates.

In conclusion, the ITC thanks Shi and colleagues for their thoughtful comments and we agree that future research is warranted to identify better probe substrates for P-gp. We encourage all academic centres and pharmaceutical companies to share data openly to advance the science, knowledge and tools in the area of drug transport.

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Suspense builds on anti-obesity rollercoaster ride

Despite high safety hurdles and a history of failure in the field, some drug firms remain cautiously optimistic for the next wave of experimental anti-obesity agents.

Dan Jones

Drug makers chasing the potential multibillion dollar market of anti-obesity agents experienced a rollercoaster ride in 2010.

Excitement started to mount when companies — Vivus, Arena Pharmaceuticals and Orexigen Therapeutics/Takeda — submitted drugs to the US Food and Drug Administration (FDA) in late 2009 and early 2010. However, the withdrawal of Abbott's anti-obesity drug (sibutramine/Meridia) in both the European Union and the United States over cardiovascular concerns showed that the safety of such agents remained paramount. Hopes fell further when the FDA rejected the initial regulatory applications for both Vivus's combination product Qnexa (phentermine plus topiramate) and Arena's lorcaserin in October, following negative recommendations by FDA advisory panels. All eyes turned to the 7 December panel meeting on Orexigen/Takeda's Contrave (bupropion plus naltrexone), amid concerns that a negative vote would cast a dark shadow over the entire field.

Surprising some, the advisory panel recommended approval, by a vote of 13–7. Although the FDA is not due to make its decision on Contrave until the end of January 2011, the panel's positive position nonetheless gave companies working in the area reason for cautious optimism. "Had this been a negative outcome it would have been very hard to convince anyone that you'd have a chance at success with a new obesity therapy," says Thomas Hughes, President and Chief Executive Officer of Zafgen, a biotechnology company developing anti-obesity agents. "We're now quite upbeat, not just about our programme, but about the field in general."

Future fat fighters?

The field includes a host of companies with candidates at various stages of development (TABLE 1), pursuing a range of strategies for tackling obesity. Some pharmacological interventions — including Qnexa, Contrave and lorcaserin — target the central nervous system (CNS) to either reduce appetite or increase feelings of satiety (*Nature Rev. Drug Discov.* **8**, 386–398; 2009). Others attempt to decrease fat storage more directly, for instance by restoring the sensitivity of adipose tissue to weight-regulating hormonal signals.

NeuroSearch's tesofensine — a small molecule that inhibits the

reuptake of dopamine, noradrenaline and serotonin — is an example of one of the more advanced CNS-targeting anti-obesity agents. In a Phase IIb study involving 203 patients, 24 weeks of treatment with tesofensine led to placebo-adjusted weight losses of 4.5–10.6 kg, depending on the drug dose (*Lancet* **372**, 1906–1913; 2008).

Although serotonin, noradrenaline and dopamine signalling pathways are involved in weight regulation, the potential downside is that they also regulate many other processes. "Whenever you try to intercept certain pathways, there's always a price to pay," says David Lau, an obesity researcher and Professor of Medicine at the University of Calgary, Alberta, Canada. And the risk with CNS-acting agents is that the price is more likely to be in the form of neuropsychiatric side effects.

The risks of such side effects might be minimized by focusing on modulating fat metabolism rather than the CNS. In obese individuals, says Hughes, adipose tissue accumulates as a result of an imbalance between fat storage and the conversion of fat into other forms of energy that the body can use. But adipose tissue does not just provide passive fat storage — it is an active part of the weight-regulation system. So, when this system becomes disordered, as occurs in obesity, fat accumulates abnormally and the system becomes less sensitive to metabolic hormones such as insulin and leptin that normally keep individuals lean.

The most advanced of the metabolic approaches — and indeed of the entire unfiled pipeline of experimental obesity products — is Novo Nordisk's liraglutide. An analogue of the gut hormone glucagon-like peptide 1 (GLP1), liraglutide modulates insulin secretion via the GLP1 receptor and is already approved for type 2 diabetes. In a Phase II trial in 564 obese individuals without diabetes liraglutide induced a mean weight loss of 4.8–7.2 kg, compared with 2.8 kg in the placebo group (*Lancet* **374**, 1606–1616; 2009). A first 56-week Phase III trial showed that liraglutide-treated subjects lost 6 kg more than placebo-treated subjects, says the company, which has yet to release detailed trial results. Two more pivotal trials are due to start in 2011.

Peptidic agents like liraglutide do have drawbacks. For one, "they're injectable, so they're not going to be straightforward to administer to patients" says John Wilding, Head of the Department of Obesity and Endocrinology at the University of Liverpool, UK.

Two are better than one?

As for many other diseases, combinations of drugs may be necessary to tackle obesity. "A single drug that addresses a single pathway or target is unlikely to be highly effective for losing weight and keeping it off," says Timothy Garvey, Chair of the Department of Nutrition Sciences at the University of Alabama at Birmingham, USA. "We're going to need drugs that hit multiple pathways or targets, or combination drugs, to be effective."

Both Qnexa and Contrave are combinations of drugs that are already approved for other indications. "The principle is right, but I'm not convinced that Qnexa and Contrave are the best combinations," says Wilding. One promising approach, he suggests, might be to combine a CNS-acting drug with a peptide hormone.

Pursuing another approach, Amylin Pharmaceuticals/Takeda are evaluating a combination of two peptide hormone analogues — pramlintide (an analogue of amylin, which is involved in glycaemic control, that is approved for type 2 diabetes) with a proprietary leptin analogue, metreleptin. In a 28-week 608-patient Phase IIb trial, said the partners, this combination induced weight loss of around 10 kg, compared with a placebo response of around 1.8 kg.

Lessons learned

Owing to the huge population of patients who could be prescribed an obesity drug in the United States, safety is clearly particularly critical for the FDA. Indeed, lorcaserin stumbled partly owing to animal carcinogenicity data, and Qnexa was held up in part by teratogenicity concerns. Yet some obesity researchers argue that the regulators have got the risk–benefit balance wrong. "The FDA is very risk averse, and especially with regard to metabolic drugs," says Garvey. "It's a particular problem for obesity therapies because of their potentially widespread use."

In light of these concerns, Hanne Leth Hillman, Director of Investor and Capital Market Relations at NeuroSearch, stresses the need for companies to make clear to regulators how their obesity agents will be used in the real world. "It's very important that in your Phase III programme you suggest a treatment algorithm that can be used once the drug has been approved to make sure that the drug does not go out to patients who are not eligible for treatment," says Hillman — a lesson gleaned by NeuroSearch from the recent FDA hearings that has informed their development programme.

Other researchers worry that FDA advisory panellists often have a different view on what counts as efficacious compared with clinicians who treat obese patients. "Clinical pharmacologists are often unimpressed with weight loss around the 5% level, and so any safety concerns can easily be seen to outweigh the benefits," says Lau. Yet, he adds, studies have shown that 5% body weight loss translates into a 58% reduction in diabetes risk (*N. Engl. J. Med.* **344**, 1343–1350; 2001). "These are huge outcomes".

One of the biggest looming issues, however, is what kind of data are needed to demonstrate that an agent does not affect cardiovascular health, and whether a large outcomes study should be run before, or after, approval. During Orexigen/Takeda's panel meeting, plans for a post-marketing outcomes study and a broad risk evaluation and mitigation strategy (REMS) seemed to assuage panellists' fears, but only a full regulatory decision will show what the FDA's stance is. If pre-approval outcome studies are needed, however, products like liraglutide that have already had to pass the high hurdles of diabetes drug safety could have an advantage.

While Arena and Vivus consider their next steps, and Orexigen/Takeda hopes for a green light, the field remains in suspense, awaiting the rollercoaster's next rise or fall. In part, says Lau, the outcome may depend on our broader attitude towards obesity. The belief that obesity is due to a failure of will power or to laziness, and that anti-obesity drugs are a quick-fix, is unfair, he says. "The public, and health professionals outside the obesity arena, just don't recognize how difficult it is to lose weight — which is why we need new therapeutic tools to complement lifestyle changes."

Table 1 | **Selected mid- to late-stage anti-obesity drugs in the pipeline**

Name	Company	Phase	Target
Liraglutide	Novo Nordisk	III	Glucagon-like peptide 1 receptor
Velneperit	Shionogi	IIb	Neuropeptide Y and peptide YY receptors
Tesofensine	NeuroSearch	IIb	Dopamine, noradrenaline and serotonin reuptake
Zonisamide plus bupropion	Orexigen Therapeutics/Takeda	IIb	Dopamine and noradrenaline reuptake; sodium and calcium channels
Pramlintide plus metreleptin	Takeda/Amylin	IIb	Amylin and leptin receptors
Tesamorelin	Merck KGaA	II	Growth hormone-releasing hormone receptor

2010 in reflection

From health-care reform to rare diseases, Asher Mullard looks back at some of the key events and themes of 2010.

The year 2010 was yet another difficult 12 months for the pharmaceutical industry. Austerity measures around the world drove down health-care budgets, and the 'patent cliff' steepened, threatening to cost the industry another US$32 billion in sales next year alone (see page 12). As in previous years, companies responded through mergers and acquisitions, deal-making (BOX 1) and sweeping job cuts — over 50,000, according to one estimate[1]. Drug safety also remained a prominent issue: the ongoing saga of cardiovascular concerns with GlaxoSmithKline's diabetes drug rosiglitazone (Avandia), in particular, culminated with the drug being severely restricted in the United States and withdrawn in the European Union. But there were, nevertheless, some positive themes as well. Even health-care reform, a development that had been viewed as a major potential threat for industry, was not as bad as had been expected.

No pain, no gain

After months of debate, horse trading and political positioning, US President Barack Obama signed wide-ranging health-care reform into US law on 23 March 2010. The trade group Pharmaceutical Research and Manufacturers of America (PhRMA) lobbied hard to influence the scope of change, and succeeded, says Business Insights analyst George Green. "In the long term, I think the health-care reform is positive for the industry," he says. "The outcome could have been much, much worse."

In an 82-page report on the overhaul[2], Green argues that the new health-care system will cost the pharmaceutical industry $119 billion in revenue from 2010 to 2019, primarily through discounts on drugs and fees on earnings. But, he counters, these losses will be offset over the second half of the decade by increased access to 32 million patients, resulting in a net upside for the industry of $19 billion by 2019.

Although the near-term hit will hurt, he adds, the effects on research and development (R&D) will be subtle, driven primarily by changes to the future profitability of different therapeutic areas. Drugs that are subject to high pricing pressure — such as antipsychotics and antiretrovirals that will be heavily rebated by the pharmaceutical industry through Medicaid, a health-care scheme for individuals on low income — may fall out of favour. Vaccines and preventive treatments that insurers will have to provide to consumers at no out-of-pocket cost, by contrast, could receive a boost.

Key successes for the industry in the negotiations included provisions blocking Medicare, which provides federal health insurance to those aged 65 years and over, from negotiating drug prices. Three thousand small biotechnology firms also received nearly $1 billion dollars in tax credits in the first round of the newly introduced Qualifying Therapeutic Discovery Project Program. Many hope the scheme will be extended and expanded next year.

Path emerges for biosimilars

Another point scored by the industry in health-care reform came within the terms of the Biologics Price Competition and Innovation Act, which created the long-awaited abbreviated pathway for the approval of biosimilars. Although these follow-on products will eventually exert pricing pressures, the act guaranteed 12 years of data exclusivity for pioneer biologics (see page 23), whereas generics firms had been lobbying for just 5 years of protection.

The full effects of the US's biosimilar pathway, such as how widely it will open the door to competition — particularly for more complex biologics such as monoclonal antibodies — remains unclear. The US Food and Drug Administration (FDA) did, however, gather stakeholders in November to voice their views on what the route should entail[3]. Among the key questions were: are our analytical tools sufficient to establish biosimilarity on a physicochemical level for different classes of product? What type, size and number of clinical trials will be sufficient to support approvability? And how will the agency consider the potential for extrapolation of data between indications?

Many observers expect the FDA to take a case-by-case approach with flexible guidance for the different classes of biologics — much in the same way that the European Medicines Agency (EMA) has proceeded since it first introduced its abbreviated biosimilars route back in 2005 (in November 2010, the EMA's Committee for Medicinal Products for Human Use approved draft guidance specific to monoclonal antibody biosimilars; http://go.nature.com/rxFFaB). As companies wait for a clear pathway to emerge in the United States, potentially over the course of 2011, several are proceeding with plans to submit their follow-on biologic products using standard biologic license applications.

Adapting to change

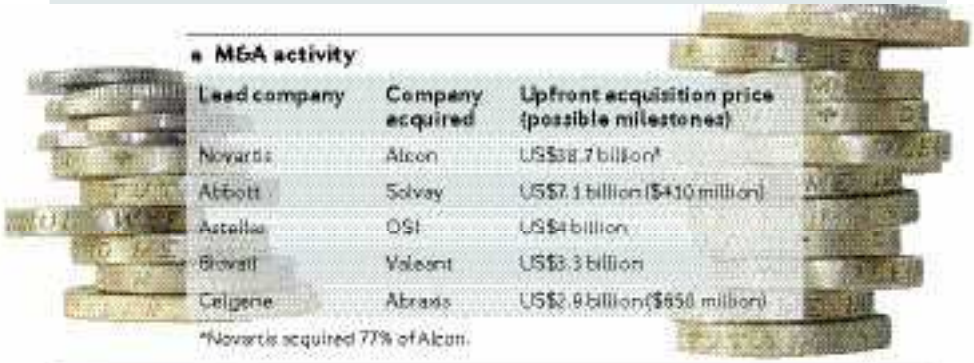
Driven in part by the FDA's publication of another draft guidance, adaptive trial design also received a lift last year. "No doubt 2010 will be regarded as a landmark in the history of adaptive clinical trials," says Donald Berry, a biostatistician at the University of Texas MD Anderson Cancer Center in Houston, USA, who has been spearheading the field of Bayesian clinical trials.

The approach provides the flexibility to modify aspects of ongoing blinded trials, such as population size or the relative allocation of a particular therapy or dose into different arms of a trial, while maintaining statistical validity. This could help accelerate trials and enable sponsors to gain information more effectively from enrolled patients. In the much-awaited draft guidance, the FDA identified key issues for consideration in the design of such trials and recommended the types of information that need to be collected and submitted to facilitate FDA review (http://go.nature.com/lpc5T8). Although the document leaves some questions unanswered, it provides a degree of certainty that observers hope will enable the field to move forward[4].

Indeed, drug makers are increasingly putting their weight behind adaptive trials. "During the year, virtually every major pharmaceutical company at least experimented with Bayesian adaptive designs, and some companies adopted this approach in a major way," says Berry. The Biomarkers Consortium, a public–private partnership that unites the US National Institutes of Health ▸

Box 1 | **Top deals**

In the hopes of softening the blow of the looming patent cliff and of bolstering weak pipelines, drug makers have continued to buy up smaller firms and to license promising drug candidates. Although the number of such transactions is down form the heady, pre-credit-crisis days of 2007, there are indications that they are on the rise[16]. BioMedTracker analysts prepared a list of the top merger and acquisition (M&A) and licensing activity (see part **a** and **b** of the figure, respectively) in 2010. Deals that are still being negotiated, such as Sanofi–Aventis's bid for Genzyme and Johnson & Johnson's bid for Crucell, are not included in the list.



a M&A activity

Lead company	Company acquired	Upfront acquisition price (possible milestones)
Novartis	Alcon	US$38.7 billion*
Abbott	Solvay	US$7.1 billion ($410 million)
Astellas	OSI	US$4 billion
Biovail	Valeant	US$3.3 billion
Celgene	Abraxis	US$2.9 billion ($650 million)

*Novartis acquired 77% of Alcon.

b Licensing deals

Lead company	Licensing company	Product (indications)	US biodollars (upfront; possible milestones)
Rigel	AstraZeneca	Fostamatinib (RA, oncology, SLE, ITP)	$1.24 billion ($100 million; $1.145 billion)
TransTech	Forest Laboratories	TTP399 (type 2 diabetes)	$1.155 billion ($50 million; $1.105 billion)
Orexigen Therapeutics	Takeda	Bupropion plus naltrexone (obesity)	$1.05 billion ($50 million; $1 billion)
Synosia	UCB	Cridadin (Parkinson's disease)	$745 million ($20 million; $725 million)
Quark	Novartis	QPI-1002 (renal disease/renal failure, kidney transplant rejection)	$680 million ($10 million; $670 million)

ITP, idiopathic thrombocytopaenic purpura; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

and pharmaceutical companies, for instance initiated the headline-grabbing I-SPY2 breast cancer trial in March. Using a combination of both biomarkers and adaptive trial design principles, it will test five drugs from three manufacturers — Abbott's veliparib, Amgen's conatumumab and AMG386, and Pfizer's figitumumab and neratinib — as adjuncts to chemotherapy. Confidence in the potential of such strategies was also raised in April by data from the BATTLE trial, which showed that biomarkers can be used in an adaptive design context to guide the treatment of patients with non-small cell lung cancer (NSCLC)[5].

Racing to the market

Some of the year's most groundbreaking clinical results also came from other oncology trials. Pfizer's crizotinib, in particular, made waves. Initially developed as an inhibitor of the tyrosine kinase MET, crizotinib also acts against ALK. When genetic rearrangements in *ALK* were reported in some lung cancers in 2007, researchers decided to test the drug in a genetically selected population. As reported in the *New England Journal of Medicine* in October, around 90% of crizotinib-treated *ALK*-positive patients with NSCLC experienced either an overall response (57%) or stable disease (33%), compared with 10% of patients in historical controls[6]. These Phase I results prompted the initiation of a Phase III trial. Also, Bristol-Myers Squibb subsidiary Medarex's immunotherapy ipilimumab, a monoclonal antibody against cytotoxic T lymphocyte-associated antigen 4, attracted attention, demonstrating impressive survival data in a Phase III advanced melanoma trial (see page 10).

Following Phase III trial successes, telaprevir and boceprevir — two members of a new class of oral drugs for hepatitis C virus (HCV) that inhibit the viral NS3 protease — are racing towards approval in 2011 for a possible multibillion dollar market. Because the current standard HCV treatment — 48-weeks on the generic antiviral ribavirin and interferon conjugated to polyethylene glycol — has only a 50% cure rate and an arduous side-effect profile, there are high hopes for these drugs[7]. Both telaprevir and boceprevir (developed by Vertex/Tibotec and Merck, respectively) performed well in treatment-naive populations (eliciting sustained virologic response (SVR) rates of 63–75%) and in treatment-experienced patients (SVR rates of 59–66%).

The race to develop alternatives to the oral anticoagulant warfarin, another potential multibillion dollar market, also heated up in 2010. Warfarin has been the standard of care for a range of indications, such as stroke prevention in atrial fibrillation (SPAF), for many years, but its use is limited by a narrow therapeutic window and variability in treatment response, necessitating regular monitoring. Consequently, several companies have been developing simpler alternatives, with SPAF considered as a key market[8].

October 2010 saw the FDA approval of one of these drugs — Boehringer Ingelheim's direct thrombin inhibitor dabigatran (Pradaxa) — making it the first new oral anticoagulant to reach the US market in more than 50 years. Dabigitran, which is already approved in Europe for the prevention of venous thromboembolism (VTE) following orthopaedic surgery, was approved in the United States for SPAF based on the 18,113-patient RE-LY trial, reported in 2009, which showed that it was non-inferior to warfarin[9]. Data from other contenders are keeping the competition interesting, however. In November, Bayer/Johnson & Johnson's pivotal ROCKET-AF trial showed that the oral factor Xa inhibitor rivaroxaban (Xarelto) is also non-inferior to warfarin. Rivaroxaban is also already approved in the European Union for the prevention of VTE following orthopaedic surgery (see page 61), and seems on track for SPAF approval in the United States in 2011. Results from a third would-be competitor, Bristol-Myers Squibb/Pfizer's oral factor Xa inhibitor apixaban, are due to be presented in August 2011 at the European Society of Cardiology meeting in Paris, France.

Another closely watched competition to introduce a pioneering oral drug — in this case, for patients with multiple sclerosis — was won by Novartis's fingolimod (Gilenya), a sphingosine 1-phosphate receptor modulator. Fingolimod was approved by the FDA in September (see next month's in-depth analysis of FDA approvals in 2010).

The push to bring a new obesity drug to market, by contrast, brought predominantly disappointment, although there is some cause for cautious optimism (see page 5). Two candidates stumbled at the hurdle of FDA

review, and then an advisory panel endorsed Orexigen Therapeutics/Takeda's bupropion plus naltrexone combination. A decision on the anti-obesity drug is due in 2011.

Notable, expensive, flops in the clinic in 2010 (BOX 2) included Medivation/Pfizer's dimebon for moderate to severe Alzheimer's disease (AD) — which may have contributed to Pfizer's CEO Jeff Kindler's unexpected resignation — and Eli Lilly & Company's semagacestat for AD. A string of failures in AD has raised broader questions about the prevailing amyloid hypothesis of this disease, on which semagacestat was based[10].

Common interests in rare diseases

While blockbuster indications like AD remain on the radar for drug makers, companies have continued to ramp up their commitment to niche busters for rare diseases over the course of 2010 as well. Pfizer, following on from its 2009 acquisition of the rights to Protalix's taliglucerase alfa for Gaucher's disease, for example, created a small R&D unit dedicated to rare diseases[11]. Pfizer also acquired FoldRx, who focus on therapeutics for protein misfolding diseases like transthyretin amyloid polyneuropathy[12].

Regulatory and financial incentives, such as those provided by the US Orphan Drug Act of 1983 and comparable legislation in Europe enacted a decade ago, have also had a key role in increasing interest in the field. Writing in a recent commentary piece[13], Timothy Coté, Director of the US FDA's Office of the Orphan Products Development, argued that further developments, including comprehensive analysis of rare disease review and regulation, "are poised to increase the momentum of rare disease R&D". It is no surprise, then, that Pfizer is not the only big pharmaceutical company striving for a piece of the pie. Sanofi–Aventis, for instance, is engaged in a long-running bid to acquire the biotechnology company Genzyme, which has built its business model on a host of drugs for rare diseases. And following the establishment of a rare diseases unit in 2010, GlaxoSmithKline recently formed an alliance with Fondazione Telethon and Fondazione San Raffaele to develop novel gene therapy approaches for rare disorders such as adenosine deaminase-severe combined immune deficiency, Wiskott–Aldrich syndrome (WAS) and β-thalassaemia.

2011: gene therapy revival?

In addition to GlaxoSmithKline's recent deal, a cluster of clinical data in 2010 indicated that gene therapy could be on the up, after a decade of disappointments and setbacks. For example, lentiviral delivery of the β-globin gene via autologous haematopoietic stem cell transfusion proved safe and effective in one patient with severe β^{E}/β^{0}-thalassaemia[14]. Retroviral delivery of the *WAS* gene using the same strategy improved the symptoms of WAS in two patients for up to 3 years, with no treatment-related adverse events[15]. And in a larger 39-patient placebo-controlled Phase II study, presented at the 2010 American Heart Association meeting in Chicago, USA, Celladon's adeno-associated virus Mydicar (sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (*SERCA2a*) gene therapy) met its primary safety and efficacy end points, including incidence of fatal and non-fatal cardiovascular events, in patients with advanced heart failure.

Although such results are encouraging, regulatory approval remains an unachieved goal. Leading the charge for change, potentially in 2011, is Amsterdam Molecular Therapeutics (AMT), who has filed Glybera (lipoprotein lipase (*LPL*) gene therapy) for the treatment of LPL deficiency in the European Union. AMT is anticipating a decision from the regulators in 2011. "It would be a landmark event to have the first gene therapy approved, for any indication," said Jean Bennett, a professor of ophthalmology who works on gene therapy at the University of Pennsylvania, USA. "I hope they can do it."

Box 2 | **Top flops**

As ever, the high attrition rate in late-stage drug development took its toll in 2010. Thomson Reuters Life Science Consulting compiled its list of the top flops of 2010, as ranked by loss of risk-adjusted consensus-analyst forecasted sales over the next 20 years (see the table). The top 10 failures — including drugs that have been discontinued and those that failed pivotal trials or face possibly surmountable regulatory setbacks — are anticipated to cost the industry nearly US$74 billion (lost revenue relates only to the indications in which the drug failed, and some of the listed drugs are still in development for other indications). Despite the hold-ups, some of these products could still make it to market. Thomson Reuters' CMR International estimates that the sunk cost of late-stage failure was approximately $300–500 million per project.

Company	Product*	Indication	Status	Lost revenue‡
Roche/ Biogen Idec	Ocrelizumab	RA/lupus	Discontinued	$13 billion
AstraZeneca	Motavizumab	RSV	CRL; another trial requested	$13 billion
Sanofi–Aventis	NV1FGF	PVD	Discontinued	$11 billion
AstraZeneca	Zibotentan	Prostate cancer	Pivotal trail failed; two more trials ongoing	$11 billion
Merck & Co	Vicriviroc	HIV	Discontinued	$10 billion
Roche/Ipsen	Taspoglutide	Type 2 diabetes	Pivotal trials halted on safety concerns	$4.8 billion
AstraZeneca	Cediranib	Cancer	Discontinued	$4.4 billion
Eli Lilly & Company	Semagacestat	Alzheimer's disease	Discontinued	$3.9 billion
Novartis/ Antisoma	ASA404	NSCLC	Discontinued	$1.8 billion
Pfizer/ Medivation	Dimebon	Moderate to severe Alzheimer's disease	Discontinued	$0.8 billion

*Drugs may still be in development for other indications. ‡Forecasted lost revenue over 20 years (US$, risk adjusted). CRL, complete response letter; NSCLC, non-small cell lung cancer; PVD, peripheral arterial disease; RA, rheumatoid arthritis; RSV, respiratory syncytial virus.

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BIOBUSINESS BRIEFS

MARKET WATCH

Upcoming market catalysts in Q1 2011

Several orphan drugs have exciting catalysts in the first quarter of 2011. These include Prescription Drug User Fee Act (PDUFA) action dates for ipilimumab for pretreated metastatic melanoma and vandetanib for medullary thyroid cancer (MTC) as well as results from the second Phase III trial of teduglutide for short bowel syndrome (SBS).

A decision by the US Food and Drug Administration (FDA) on the approval of ipilimumab — a fully human monoclonal antibody developed by Medarex (now part of Bristol-Myers Squibb) that targets the immune-cell receptor CTLA4 (cytotoxic T lymphocyte-associated antigen 4) to block the T-cell inhibitory pathway — is expected by 26 March. Ipilimumab was developed as a monotherapy for advanced melanoma in patients who have received prior therapy, for whom the median overall survival is 6 to 9 months. The drug failed to meet the end point of an objective response rate of more than 10% in a pivotal study. Medarex subsequently met with the FDA and the agency requested additional overall survival data to further determine the benefit of ipilimumab.

Recent results from a Phase III trial of ipilimumab as a monotherapy or in combination with a gp100 peptide vaccine demonstrated a median overall survival of 10.1 months and 10.0 months, respectively (*N. Engl. J. Med.* **363**, 711–723; 2010). Although some significant toxicities were noted with treatment, given the impressive efficacy in increasing survival, ipilimumab is poised to become the first approved agent for metastatic melanoma.

An FDA decision is also anticipated soon on vandetanib, developed by AstraZeneca, for the treatment of patients with unresectable locally advanced or metastatic MTC, which accounts for 5% of all thyroid cancers and is incurable. Vandetanib is a multitargeted kinase inhibitor exhibiting potent activity against vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and rearranged during transfection (RET) pathways.

Phase III results showed that treatment with vandetanib significantly extended progression-free survival, demonstrating a 54% reduction in the rate of progression compared to placebo. Significant differences for vandetanib compared to placebo were also observed in objective response rate and disease control rate. Given these results, the drug's acceptable safety profile, and a positive outcome at an FDA advisory committee meeting on 2 December, vandetanib seems to be in a good position to win approval in January 2011.

Finally, data are expected from a confirmatory trial of teduglutide (developed by NPS Pharmaceuticals), in patients with SBS, which is primarily caused by surgical removal of half or more of the small intestine. Parenteral nutrition (intravenous feeding) can help compensate for the reduced nutrient absorption; however, it is associated with serious complications, such as infections or liver damage.

Teduglutide is an analogue of glucagon-like peptide 2 (GLP2), a naturally occurring hormone that regulates the growth, proliferation and maintenance of the cells lining the small intestine. Results from a Phase III study showed a trend for the high dose in improvement of total parenteral nutrition (TPN) requirement, but this was not statistically significant. Although the statistical criteria were such that if the high dose failed, the low dose would not be analysed, the company went forward with the low-dose analysis, which demonstrated a significant improvement in TPN. Consequently, a second Phase III study was initiated to evaluate the low dose and to confirm the previously reported data. Positive results may enable NPS to file a regulatory application for teduglutide for adult patients with SBS who are dependent on parenteral nutrition.

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> “Given the impressive efficacy in increasing survival, ipilimumab is poised to become the first approved agent for metastatic melanoma.”

BIOBUSINESS BRIEFS

TRIAL WATCH

Hope renewed for strategy to raise HDL cholesterol

> By helping define the mechanism underlying the benefit of HDL… the coming clinical trials and basic science research will go a long way towards solving the enduring mysteries of HDL.

Positive results of the Phase II DEFINE trial of Merck's cholesteryl ester transfer protein (CETP) inhibitor anacetrapib (*N. Engl. J. Med.* 17 Nov 2010) have revitalized confidence in the potential of this approach to reduce heart disease risk, which was severely dented by the dramatic failure of Pfizer's torcetrapib in 2006. "These exciting results indicate a green light for proceeding with a large clinical outcomes study", says Alan Tall of Columbia University, New York.

Lipid abnormalities, particularly elevated low-density lipoprotein (LDL) cholesterol and reduced high-density lipoprotein (HDL) cholesterol, are major risk factors for the development of cardiovascular disease. Although statins (which lower LDL cholesterol) have proved to be highly effective at reducing such risk, there are apparent limits to the degree of benefit attainable from targeting LDL cholesterol alone. These limits have stimulated investigations into strategies to normalize additional lipid abnormalities, such as raising HDL cholesterol (*Nature Rev. Drug Discov.* **4**, 193–205; 2005).

One such strategy is to inhibit CETP, which binds to HDL in the circulation and exchanges HDL cholesteryl esters with triglycerides from very-low-density lipoprotein (VLDL) cholesterol during reverse cholesterol transport. This approach was suggested by the finding that decreased CETP levels — due to *CETP* mutations — are associated with increased levels of HDL cholesterol and reduced levels of VLDL and LDL cholesterol. However, in 2006 a 15,000-patient Phase III trial of the CETP inhibitor torcetrapib was terminated early owing to increased deaths and cardiovascular events (*Nature Rev. Drug Discov.* **7**, 143–155; 2008), casting doubt over the entire field.

DEFINE was therefore designed to assess the side effects and overall safety profile of anacetrapib, as well as its effects on lipid levels. The trial involved 1,623 patients with or at high risk for coronary heart disease and currently undergoing statin therapy. The results are promising. "The trial indicates with at least 94% probability that anacetrapib carries less risk of adverse effects compared with torcetrapib, albeit over a relatively short time period", notes Rob Hegele, Robarts Research Institute, Ontario.

Importantly, "there appears to be some mechanistic daylight between anacetrapib and torcetrapib", says Hegele. As Tall notes, "torcetrapib raised blood pressure and sodium levels while lowering potassium levels, which may have been related to hyperaldosteronism or increased production of other adrenal cortical steroids. None of this has been seen with anacetrapib. There have been concerns that CETP inhibition itself might not be good for heart disease, but these [concerns] should now be allayed."

"Perhaps more surprising are the efficacy data", says Hegele. "Not only was the HDL cholesterol increase larger than with almost any other therapy (~138%), the drug also markedly lowered LDL cholesterol (~40%)." However, he adds that "while it is accepted that lowering LDL cholesterol by any means reduces cardiovascular risk, there remain lingering doubts that raising HDL quantity alone without considering its quality or functional behaviour will necessarily show benefits. This theoretical advantage of increased HDL will therefore need to be defined with a large clinical outcomes study."

Such a study for anacetrapib, involving ~30,000 patients, is anticipated to begin in the first half of 2011. A Phase III trial of another CETP inhibitor, Roche's dalcetrapib, is ongoing, with results expected in 2013. Other approaches for targeting HDL, including niacin and elevating levels of apolipoprotein A1 — the major protein component of HDL — are also under clinical investigation. "By helping define the mechanism underlying the benefit of HDL, whether it is reverse cholesterol transport from the artery wall or another mechanism, such as suppression of inflammation or improvement in vasoreactivity, the coming clinical trials and basic science research will go a long way towards solving the enduring mysteries of HDL", concludes Hegele.

PATENT WATCH

The patent cliff steepens

Many blockbuster drugs — including Lipitor (atorvastatin; Pfizer), Plavix (clopidogrel; Sanofi–Aventis/Bristol-Myers Squibb) and Zyprexa (olanzapine; Eli Lilly & Company) — are facing expiry of their US patents in the next 2 years, which is expected to lead to plummeting sales owing to competition from generic versions of these drugs.

"The patent cliff facing the industry is very real, with billions of dollars being stripped from companies' revenues," says Michael Hay, an analyst at Sagient Research Systems. "Although the vast majority of the drugs losing patent protection are sold by large pharmaceutical companies that are well diversified, the amount of revenue that will be lost is going to be very difficult to make up for," he adds (see FIG. 1).

Indeed, the impending patent cliff is anticipated to erode US$78 billion in worldwide sales from branded drugs that are facing patent expiry between 2010 and 2014 (REF. 1), with nearly half of this erosion expected to occur owing to the loss of patents in 2011 for major blockbuster drugs.

The most prominent drug to lose US patent protection in 2011 is the world's top-selling drug: Pfizer's Lipitor, a cholesterol-lowering drug in the statin class that brought the company more than $11 billion in revenue in 2009 (TABLE 1). Lipitor accounts for over 20% of Pfizer's total revenue, and the company has no drugs in late-stage development that look likely to replace the revenue that will be lost. The loss of patent protection for Lipitor will also harm sales of other branded products in the antidyslipidaemia market.

"The availability of generic atorvastatin will provide health-care payers with another price-discounted option in addition to statins such as simvastatin for managing cholesterol levels in patients," says Gideon Heap, an analyst at Datamonitor Healthcare. "Growth of the antidyslipidaemic market will end when Lipitor loses patent protection in the United States. However, AstraZeneca have positioned their statin Crestor [rosuvastatin; patented until 2016, which had $4.5 billion in sales in 2009] to survive the genericization of the statin market through the well-designed GALAXY programme of clinical trials. In particular, the JUPITER trial has led to the approval of Crestor in a preventive indication in which other statins have not been approved, which has driven Crestor to gain share from other statins."

Also in the cardiovascular disease area, Sanofi–Aventis is facing the loss of patent protection for two blockbuster anticoagulants in the United States in 2012: Lovenox (enoxaparin) and Plavix (co-marketed with Bristol-Myers Squibb). "Both drugs have exceptionally broad clinical applications and have become dominating forces in the cardiovascular world," comments Jonathan Angell, an analyst at Datamonitor. Over the next 10 years, Sanofi–Aventis is predicted to lose $9 billion in revenue owing to competition from generic versions of these products.

> "The impending patent cliff is anticipated to erode US$78 billion in worldwide sales from branded drugs that are facing patent expiry between 2010 and 2014."

"However, the company's plan to replace Plavix and Lovenox with a range of drugs each with distinctly more focused roles, such as Multaq (dronedarone, a potassium channel blocker), idrabiotaparinux (an indirect factor Xa inhibitor) and semuloparin (an ultra-low molecular weight heparin), has not been as successful as hoped — adding to the pressure on Sanofi–Aventis to in-fill its pipeline through acquisition," says Angell. "The ramifications of generic versions of Lovenox and Plavix are far-reaching," Angell continues. "Given the volume of the prescriptions of these products, price reductions will substantially raise the bar with regard to cost-effectiveness debates for new market entrants. This compounds the challenge for such entrants in a market long dominated (in volume terms) by aspirin and warfarin, which in 2008 accounted for 65% of the market by volume, but just 7% by value."

The market-leading product for diabetes in 2009, Takeda's thiazolidinedione Actos

Table 1 | **Selected drugs facing patent expiry in the United States**

Branded drug (INN drug name; company)	Indication	Worldwide 2009 sales (billion)*	Expected patent expiry
Aricept (donepezil; Eisai/Pfizer)	Alzheimer's-type dementia	¥303.8 (US$3.61)	Nov 2010
Lipitor (atorvastatin; Pfizer)	High cholesterol	US$11.43	2011
Zyprexa (olanzapine; Eli Lilly & Company)	Schizophrenia, bipolar I disorder	US$4.92	2011
Lexapro (escitalopram; Forest Laboratories/ Lundbeck)	Depression and anxiety	DKK 7.77 (US$1.37)	2012
Actos (pioglitazone; Takeda)	Type 2 diabetes	¥334.5 (US$3.98)‡	2012
Plavix (clopidogrel; Sanofi–Aventis/Bristol-Myers Squibb	Clot-related cardiovascular events	US$6.15	2012
Lovenox (enoxaparin; Sanofi–Aventis)	Acute deep vein thrombosis	€3.04 ($4.03)	2012
Seroquel (quetiapine; AstraZeneca)	Schizophrenia, bipolar disorder, major depressive disorder	US$4.87	2012

*Data from company annual reports. ‡Europe and the Americas. INN, international nonproprietary name.

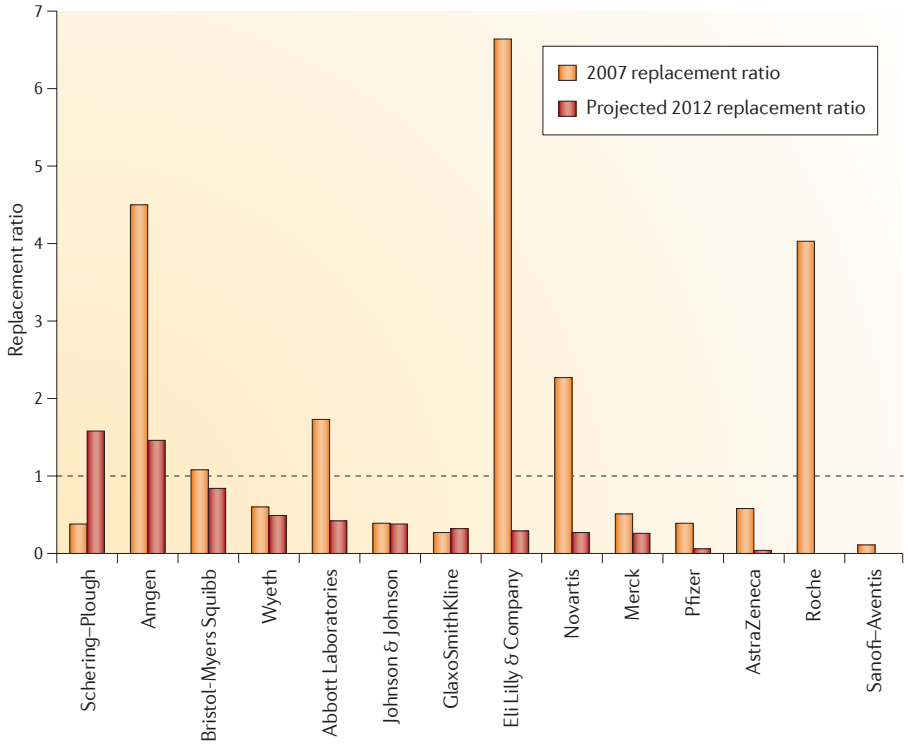
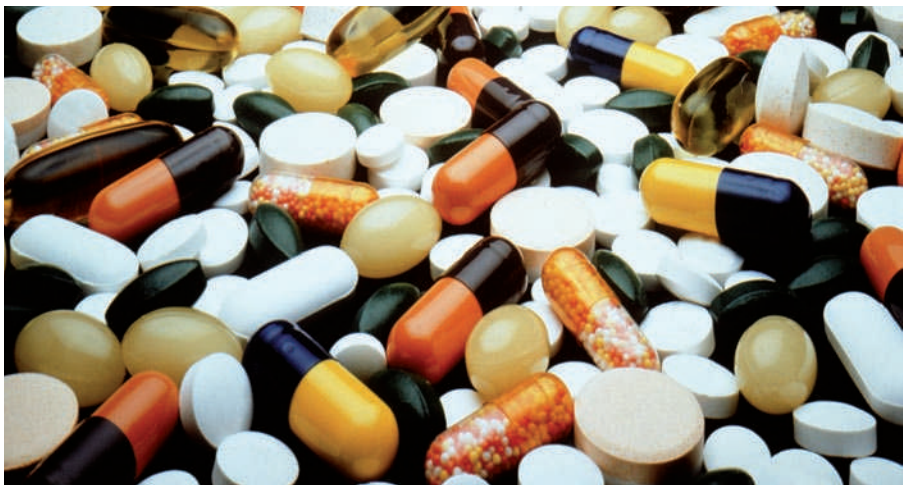


Figure 1 | **Projected replacement ratios for large pharmaceutical companies.** The replacement ratio in a given year is the ratio of revenue from new products (that is, those launched in the previous 5 years) to the revenue lost from declining products (for example, owing to generic competition). This ratio is a measure of research and development sufficiency; a ratio of less than 1 reflects a failure to replace former successful products with new revenue drivers. The data, which are taken from REF. 2, are based on each company's major prescription drug portfolio (the collection of branded drugs each of which is projected to achieve at least US$500 million in annual sales), and were compiled prior to the mergers of Pfizer and Wyeth, and Merck and Schering–Plough.

(pioglitazone), is also facing imminent loss of patent protection. "The patent expiry of Actos in 2011 will see a flood of cheap generic alternatives," says Christine Henry, an analyst at Datamonitor. "Despite this, the patent cliff will have less impact in diabetes than in many other markets, as newer drugs, [such as the dipeptidyl peptidase 4 (DPP4) inhibitor sitagliptin (Januvia; Merck & Co)] and a growing diabetic population will drive market growth from 2012 onwards," she says. "Also, given the concerns about the cardiovascular safety of the thiazolidinedione class — in particular rosiglitazone (Avandia; GlaxoSmithKline), [the use of which is restricted in the United States] — generic pioglitazone is less likely to have a significant impact on other classes of branded antidiabetics."



One commonly pursued strategy by which companies can try to compensate for patent loss is to extend the life cycle of the branded product through reformulations. For example, in view of Aricept (donepezil; Eisai/Pfizer), an acetylcholinesterase inhibitor used for the treatment of Alzheimer's-type dementia, losing its patent protection in November 2010, Eisai developed two reformulations: a sustained release oral formulation and a once-weekly transdermal patch.

However, according to Daniel Chancellor of Datamonitor: "The small time frame in which the company has had to switch patients to the other formulations, competition that the patch will experience from the well-established Exelon patch (rivastigmine; Novartis) and competition from generic versions of donepezil means that the reformulations are unlikely to be successful in maintaining Eisai/Pfizer's share of the Alzheimer's market in the United States."

Although the decline of the revenue from the Aricept franchise is anticipated to be steep (US Alzheimer's-specific revenues of Aricept formulations are expected to decline to $285 million by 2019), the Alzheimer's disease market in the United States is predicted to grow, says Chancellor. "This growth — to $6.4 billion by 2019 — will be predominantly driven by the prescribing of new disease-modifying drugs such as bapineuzumab (Elan/Johnson & Johnson/Pfizer), solanezumab (Eli Lilly & Company) and Gammagard (intravenous immunoglobulin; Baxter), which, if successful in ongoing Phase III trials, are anticipated to launch in the United States from 2013."

However, as Hay concludes, "if companies are unable to bring new drugs to market they will either need to cut spending to maintain profit or acquire new drugs that are generating sales, through mergers and acquisitions. But given the scale of revenue being lost, it is difficult and expensive to gain enough revenue through the latter route."

Charlotte Harrison

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AN AUDIENCE WITH…

Francis Collins

When Francis Collins was appointed as Director of the US National Institutes of Health (NIH) last year, he listed translational medicine as one of his top five priorities. Although some elements of this push were already in place, others remain to be implemented. Taken together, he hopes these efforts will both 'de-risk' drug development and empower academic researchers to become better partners for industry. **Asher Mullard** caught up with Collins to discuss the industry's woes and how the NIH can help.

Why did you prioritize translational medicine?

We have seen a deluge of new discoveries in the last few years on the molecular basis of disease. This is true for rare diseases, common diseases and neglected diseases, and allows us to feed new ideas into the therapeutic pipeline. That's the good news. But there is bad news too. Despite increasing investments by the private sector, there has been a downturn in the number of approved new molecular entities over the last few years. Also, drug development research remains very expensive and the failure rate is extremely high.

Perhaps in part responding to these factors, and to the downturn in the economy, pharmaceutical companies have cut back their investments in research and development. We can't count on the biotech community to step in and fill that void either, because they are hurting from an absence of long-term venture capital support. So, we have this paradox: we have a great opportunity to develop truly new therapeutic approaches, but are undergoing a real constriction of the pipeline. One solution is to come up with a non-traditional way of fostering drug development — through increased NIH involvement.

How do you plan to do this?

I like to think of this in a broad sense of "what kind of paradigm can we initiate and expand between academic researchers and the private sector to move the therapeutic agenda forward?" Academic investigators have always played some role in drug development, but usually in the earliest stages of target identification. If we want to see those targets exploited — recognizing that many of them are not initially attractive economically because of their uncertain druggability or perhaps relevance for only a rare disease — then academic investigators need to have the tools to push discovery efforts forward themselves.

By having the NIH more engaged in the pipeline, we can also ask whether we can improve the success rates of drug development. Pharmaceutical companies have been making drugs for a long time, and have created some great products, but there's been less consideration of the whole drug development pipeline itself as a scientific problem. We need to re-engineer the process, with a lot more focus on the front end.

What programmes do you have in place?

We have several different programmes that we are working to fit together. We have four NIH-funded facilities that collectively have the capacity of a midsized pharmaceutical company to do high-throughput screening, assay development and medicinal chemistry. In the preclinical space — moving promising compounds through the expensive and risky 'valley of death' — Therapeutics for Rare and Neglected Diseases (TRND) supports projects that would not be of interest to commercial players because of modest market sizes. The Cures Acceleration Network (CAN), the newest arrival on the scene, will also support preclinical 'high need' research, defined pretty much as any area where therapeutics are lacking and in need of development: it is not limited to rare and neglected diseases, and could also support neglected targets. CAN was created as part of the Healthcare Reform Act, though we are still awaiting funding approval for the 2011 financial year. The Clinical and Translational Science Awards (CTSA) and the NIH Clinical Center then provide broad support by empowering academics to run Phase I and II trials.

Another opportunity we are talking about is to pay particular attention to compounds that have been extensively studied by pharmaceutical firms but have for some reason been abandoned. Companies have been reluctant to open their freezers to us in the past, but are now much more interested in doing so.

There are a lot of moving parts to this set of resources that ultimately need to be synthesized into a smooth process. One of my goals over the next year is to try to identify ways to put these together into a more seamless enterprise.

How is the NIH's relationship with the US Food and Drug Administration (FDA) evolving as you pursue drug development?

Peggy Hamburg and I started talking about the need for tighter collaboration between the NIH and the FDA even before I was formally appointed. And we've now set up an NIH–FDA Leadership Council to focus on improving the prospects for getting effective and safe drugs to market as quickly as possible. A lot of the NIH investigators who are being empowered to conduct drug development work aren't that familiar with how the FDA does business — I hope that by working more closely together researchers will have greater understanding of what they need to do so that they don't stub their toes and then have to backtrack to meet FDA standards.

Drug discovery is a risky, expensive activity. How will the NIH be compensated for its efforts?

Our approach will be to 'de-risk' projects that might otherwise be seen as economically unattractive. As soon as the risks are reduced sufficiently to attract commercial attention, we plan to hand over projects to companies to carry out the next step. There is absolutely no intention of turning the NIH and its grantees into competitors with the private sector. We are aware of just how risky this approach is: most projects will fail and we will not reap rewards overnight. But in projects where the NIH has invested a lot of the upfront effort, there will be a model — which pharmaceutical companies seem pretty comfortable with — for sharing intellectual property rights in a way that royalties will flow back into public research. I think that's only fair.

FROM THE ANALYST'S COUCH

Data exclusivity for biologics

Henry Grabowski, Genia Long and Richard Mortimer

PANNA chair by Tokujin Yoshioka for Moroso. Photographed by Alessandro Paderni

As part of the recently enacted health-care reform legislation in the United States, the US Congress authorized an abbreviated regulatory pathway for the approval of biosimilars (also often described as follow-on biologics)[1]. Among other provisions, the legislation grants a new innovative biologic (termed pioneer biologic here) 12 years of data exclusivity, with the potential for an extension of 6 months if paediatric studies are conducted.

Data exclusivity is the length of time before a biosimilar can receive approval from the US Food and Drug Administration by relying at least in part on the safety and efficacy data for the pioneer biologic. Data exclusivity is one factor contributing to market exclusivity — the period of time during which a therapy is the only marketed version of that molecule — but not the only determinant. The results of patent litigation, the time spent in development and regulatory review, commercial decisions by competitors, and other factors all contribute to the market exclusivity period. The appropriate length of the data exclusivity period was widely debated before the abbreviated pathway for biosimilars was enacted; indeed, the US Congress considered bills with periods ranging from 5 years to 14 years. An earlier article intended to help inform this debate developed a financial model to evaluate how long a market exclusivity period would be required until a typical pioneer biologic earned a positive investment return[2]. In this model (see Supplementary information S1 (box)) for details), a representative portfolio of pioneer biologics would be expected to 'break-even' (or to recover the average costs of development, manufacturing and promotion, and the cost of capital) in 12.9–16.2 years. Here, we present an analysis incorporating two refinements that have been made to this model that directly address important concerns that were raised during the debate leading up to the enactment of the recent US legislation.

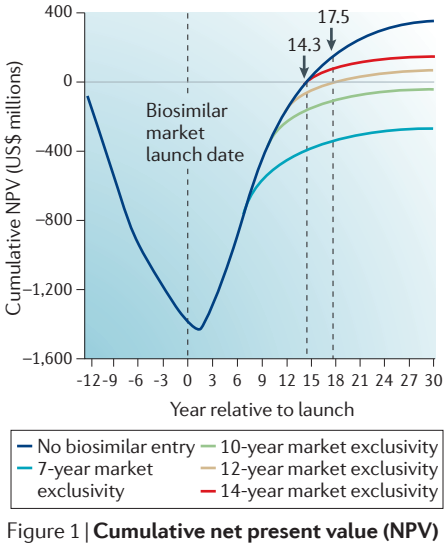

Figure 1 | **Cumulative net present value (NPV) of cash flows for a representative biologic with various scenarios for market exclusivity.** For details of the assumptions, see Supplementary information S1 (box).

Analysis

Some critics of a 12-year data exclusivity period, including the US Federal Trade Commission in a 2009 report[3], have argued that 'early mover' competitive advantages should be sufficient to maintain innovation incentives, given relatively few expected biosimilar entrants and the likelihood that biosimilars will not be interchangeable with the pioneer biologic, which is the case with generic small-molecule drugs. To investigate this issue, the original model[2] has been modified to explicitly incorporate the impact of competition between the pioneer biologic and biosimilars after market exclusivity expires. We examined how substantial retention of sales for the pioneer biologic after biosimilar entry affects the break-even lifetimes for innovators. In addition, the extent to which patents provide protection against the early entry of competitors to a pioneer biologic was also debated. We have therefore conducted a simulation analysis examining interactions between data exclusivity and patent protection (each of which contribute to market exclusivity periods) in different scenarios to highlight the specific circumstances in which each of these modes of protection is important in maintaining innovation incentives.

An analysis of cumulative net present value for a representative pioneer biologic over its life cycle is presented in FIG. 1. This analysis incorporates the research and development (R&D) and sales information of the representative portfolio of pioneer biologics examined in the previous article[2]. It also incorporates a cost of capital of 12% and a contribution margin on sales of 50%, consistent with the earlier study. However, unlike the previous model, the analysis also explicitly models the impact of biosimilar entry on the market share for the pioneer biologic, assuming that the market share for the biosimilar reaches 50% by year 4 following its entry and price discounts for the pioneer biologic reaching 15%, partially matching biosimilar discounts (Supplementary information S1 (box)). Examining how various market exclusivity periods affect break-even lifetimes, we found that the representative pioneer biologic fails to break-even under both 7-year and 10-year market exclusivity periods, even assuming it retains substantial market share after biosimilar entry. Break-even does occur with 12-year and 14-year market exclusivity periods, taking 17 years and 15 years, respectively. This compares to a break-even period of 14 years in the case of no biosimilar entry.

Although FIG. 1 underscores the impact of at least a 12-year market exclusivity period, it does not distinguish between the contributions of patent protection and data exclusivity in achieving this outcome. After market launch, data exclusivity and patent protection run concurrently. Data exclusivity provides additional market exclusivity protection only to the extent that patents can be circumvented by a biosimilar, or the remaining patent protection is shorter than the data exclusivity at the time of approval of the pioneer biologic.

To distinguish between the effects of these two modes of protection, we conducted a Monte Carlo simulation analysis, in which we defined market exclusivity as the longer of the data exclusivity and patent protection periods. In particular, we considered the effects of the different data exclusivity periods on break-even outcomes under alternative assumptions about patent protection. In scenario 1 (strong patent protection), biologic patents provided a lengthy expected period of protection against biosimilar entry (14 years on average). In scenario 2 (limited patent protection), we assumed only 7 years of expected patent protection, reflecting a lengthy R&D period or the possibility of successful patent challenges. Our analysis is based on the results of 1,000 Monte Carlo simulations for each scenario and specified data exclusivity period. The simulation draws values from normal distributions of the cost of capital and ▸

DATA EXCLUSIVITY FOR BIOLOGICS | MARKET INDICATORS

contribution margin, as well as the share and price of the pioneer biologic. The mean and standard deviations for these distributions are based on values observed in various empirical analyses (Supplementary information S1 (box)). The sample means are also consistent with the average values underlying the analysis in FIG. 1. The patent protection periods in each scenario are also drawn from a normal distribution to reflect the uncertainty from patent challenges and other events.

The strong patent protection scenario presented in FIG. 2a assumes a mean of 14 years and a standard deviation of 3 years, with 95% of the draws having a patent protection period between 8 years and 20 years. In this scenario, the data exclusivity period has only a small impact on the likelihood of breaking-even and therefore on investment incentives (that is, patent protection alone is sufficient in most cases to ensure a sufficient market exclusivity period to achieve break-even status). Correspondingly, the data exclusivity period also adds little cost, as it is infrequently binding. The simulation outcomes in FIG. 2a show that patent protection alone (with no data exclusivity period) results in a 70% likelihood of breaking-even within 25 years of launch. A 12-year data exclusivity period is binding in only 24% of the draws; increases the average market exclusivity period by less than half a year, from 14.1 years to 14.5 years; and increases the likelihood that a typical biologic portfolio investment will break-even within 25 years from 70% to 75%.

The limited patent protection scenario presented in FIG. 2b assumes a mean of 7 years and a standard deviation of 2.5 years, with 95% of the draws having a patent protection period between 2 years and 12 years. In this scenario, patent protection alone (with no data exclusivity period) results in only a 14% likelihood of breaking even within 25 years of launch. A 7-year data exclusivity period is binding in almost 50% of the draws, but only increases the likelihood of breaking-even within 25 years from 14% to 17%. A 12-year data exclusivity period is almost always binding (in 97% of the draws); increases the average market exclusivity period from 7.1 years (with no data exclusivity period) to 12 years; and increases the likelihood of breaking-even within 25 years from 14% to 62%, suggesting greatly enhanced incentives for investment.

Policy implications

The results of this analysis are consistent with the US Congress's determination that a 12-year data exclusivity period for new biologics appropriately balances potential cost savings from price competition from biosimilars with long-term incentives for investment in innovative biologics. To the degree that biologic patents are relatively less certain and more vulnerable to challenge (our limited patent protection scenario), a data exclusivity period of 12 years greatly enhances investment incentives. Conversely, if biologic patents provide relatively strong protection with significant patent life remaining at approval, patents alone will be sufficient to maintain investment incentives in most cases. In those instances, the data exclusivity period has only a minimal effect on market exclusivity times and thus on health-care costs. The 12-year data exclusivity period therefore operates mainly as an 'insurance policy' to encourage innovation when patent protection is limited.

US data exclusivity periods are now longer for biologics than for new chemical entities (NCEs). Under the Hatch–Waxman legislation, the data exclusivity period is 5 years for NCEs. Patent challenges can be filed after 4 years, but face an additional stay on generic entry of up to 30 months. So, even allowing for a 30-month stay, small molecules with early patent challenges have shorter data exclusivity periods compared to biological entities (that is, 6.5 years versus 12 years). These differences, together with uncertainty on the outcomes of patent challenges early in the life cycle of a product, raise the question of whether future incentives for innovation will be tilted in the direction of biologics. This remains an important issue for further research. It is also notable that the European Union sought to avoid this outcome by harmonizing data exclusivity for both biological entities and chemical entities at 10 years plus an additional year for establishing a clinically important new indication.

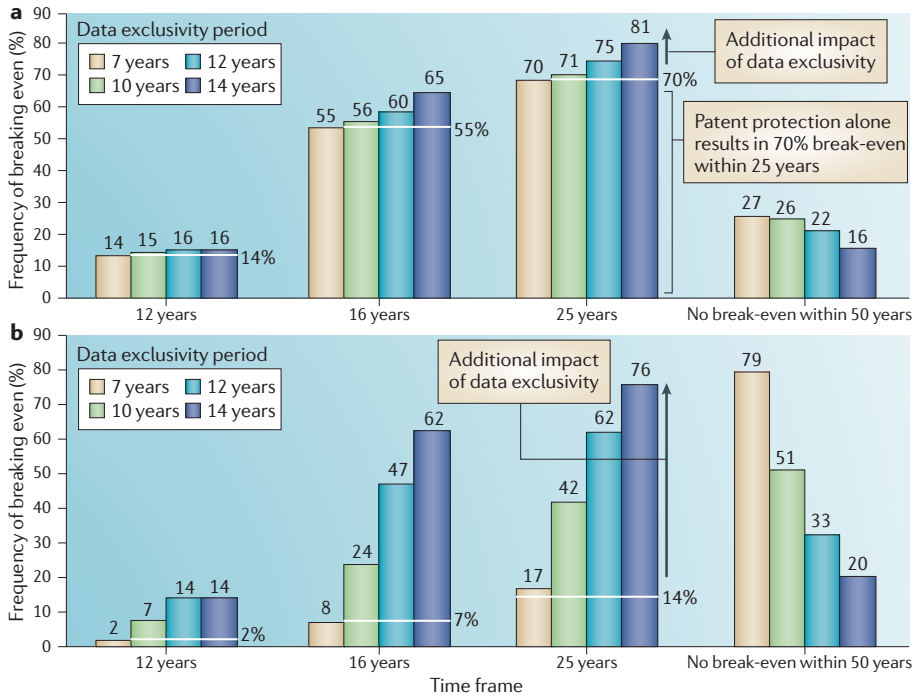

Figure 2 | **Influence of data exclusivity and patent protection on break-even points for a representative biologic portfolio.** Scenarios in parts **a** and **b** are based on strong patent protection (average period of 14 years) or limited patent protection (average period of 7 years), respectively. For details of the assumptions, see Supplementary information S1 (box).

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Competing interests statement
The authors declare no competing financial interests.

FRESH FROM THE PIPELINE

Pegloticase

Naomi Schlesinger, Uma Yasothan and Peter Kirkpatrick

In September 2010, pegloticase (Krystexxa; Savient Pharmaceuticals), a recombinant urate oxidase conjugated to polyethylene glycol (PEG), was approved by the US Food and Drug Administration (FDA) for the treatment of chronic gout in adult patients refractory to conventional therapy.

Gout, the most common inflammatory arthritis in adults, is caused by the formation of monosodium urate (MSU) crystals in joints and other tissues[1]. If untreated, tophi consisting of mononucleated and multinucleated macrophages surrounding deposits of MSU crystals can form[2]. A key risk factor for gout is chronic hyperuricaemia — a serum urate concentration exceeding the limit of solubility — and treatment approaches focus on reducing serum urate levels, as this can prevent or reverse crystal deposition[1,3]. Evidence-based recommendations[4] for the treatment of chronic gout from the European League Against Arthritis (EULAR) suggest maintaining serum urate levels below 6 mg per dL.

For several decades, allopurinol, which blocks uric acid synthesis by inhibiting xanthine oxidase (FIG. 1), has been the mainstay of urate-lowering therapy (ULT) for chronic gout[3,5]. However, not all patients receiving allopurinol achieve the desired reduction in serum urate levels, in part because some patients are not able to tolerate therapy. Febuxostat (Adenuric/Uloric; Ipsen/Menarini/Teijin Pharma/Takeda), another xanthine oxidase inhibitor that was developed with the aim of addressing the limitations of allopurinol, was granted marketing approval in the European Union in 2008 (REF. 5) and in the United States in 2009, where it has now been joined by pegloticase.

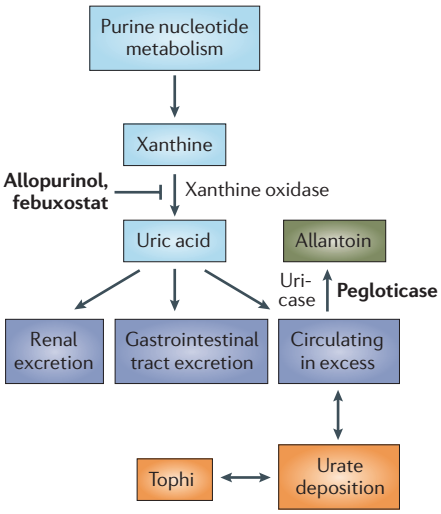

Figure 1 | **Pathogenesis of gout and targets for therapeutic intervention.** Adapted from REF. 3.

Basis of discovery

In species other than humans and some non-human primates, the enzyme urate oxidase (also known as uricase) converts urate to allantoin (FIG. 1), which is substantially more soluble and readily excreted[3]. Initial studies of a recombinant form of urate oxidase from *Aspergillus flavus,* rasburicase (Elitek/Fasturtec; Sanofi–Aventis), developed for the treatment of tumour lysis syndrome in children with cancer, indicated the potential of such enzymes to lower serum urate levels in patients with gout[3]. However, the use of rasburicase for gout is limited by its immunogenicity and a short half-life[3]. With the aim of overcoming these problems, a PEGylated recombinant mammalian uricase, pegloticase, was developed[6–8].

Drug properties

Pegloticase consists of a recombinant modified mammalian urate oxidase, produced in *Escherichia coli*, covalently conjugated to monomethoxy-PEG, of 10 kDa molecular mass[8]. It catalyses the oxidation of urate to allantoin, thereby lowering serum urate levels[8].

Clinical data

The safety and efficacy of pegloticase were studied in two 6-month, randomized, double-blind, placebo-controlled trials involving 212 adult patients with chronic gout refractory to conventional therapy[8]. Entry criteria were as follows: baseline serum urate levels of at least 8 mg per dL; symptomatic gout with at least three gout flares in the previous 18 months or at least one gout tophus or gouty arthritis; and self-reported medical contraindication to allopurinol or medical history of failure to normalize serum urate levels (to less than 6 mg per dL) with at least 3 months of allopurinol treatment at the maximum medically appropriate dose[8]. The trials were stratified for the presence of tophi; 71% of patients had tophi at baseline[8].

The patients were randomized to receive pegloticase (8 mg administered by intravenous infusion every 2 weeks or every 4 weeks), or placebo in a 2:2:1 ratio[8]. All patients also received an oral antihistamine, intravenous corticosteroids and acetaminophen as prophylaxis for infusion reactions, and non-steroidal anti-inflammatory drugs or colchicine, or both, as prophylaxis for gout flares, beginning at least 1 week before pegloticase treatment, unless medically contraindicated or not tolerated[8].

The primary end point in both trials was the proportion of patients with serum urate levels less than 6 mg per dL for at least 80% of the time during month 3 and month 6 (REF. 8). The effect of treatment on tophi after 6 months, assessed by blinded central analysis of standardized digital photographs, was a secondary efficacy end point[8]. A complete response was defined as 100% resolution of at least one target tophus, no new tophi appearing and no single tophus showing progression[8].

A greater proportion of patients receiving pegloticase every 2 weeks achieved urate lowering to below 6 mg per dL than patients receiving placebo; 20 patients (47%) in one trial and 16 patients (38%) in the other trial met this end point, compared with none of the patients receiving placebo in either trial[8]. With regard to the effect of treatment on tophi, the percentages of patients who showed a complete response after 6 months were 45%, 26% and 8% for patients receiving pegloticase every 2 weeks, every 4 weeks or placebo, respectively[8]. Treatment with pegloticase once every 4 weeks also showed efficacy with respect to the primary end point, but this regimen was associated with increased frequency of anaphylaxis and infusion reactions, as well as lower efficacy with respect to tophi[8].

Indications

Pegloticase is approved by the US FDA for the treatment of chronic gout in adult patients who are refractory to conventional therapy[8]. ▸

ANALYSIS | THERAPIES FOR GOUT

Analysing issues in the treatment of gout is Naomi Schlesinger, M.D., Chief, Division of Rheumatology, Department of Medicine, Robert Wood Johnson University Hospital, New Jersey, USA.

Gout is caused by the deposition of MSU, with the amount of tissue deposition depending on susceptibility, the length of time the patient has been hyperuricaemic and the total body uric acid pool, which is represented by the serum urate level. Persistent low-grade inflammation is frequently present in patients with asymptomatic chronic tophaceous gout[9]. Chronic treatment of gout therefore includes ULT and anti-inflammatory prophylaxis. The goal of ULT is to reduce serum urate levels to below the saturation threshold (6.8 mg per dL) for a long enough period to allow dissolution of tissue MSU crystal deposition.

The recent FDA approval of pegloticase has now provided the first treatment for patients with symptomatic gout for whom current ULTs are ineffective or are contraindicated (largely due to the presence of co-morbidities). It is notable that 71% of the patients in the pivotal trials of pegloticase severely affected by treatment failure had tophi at baseline[8]. Key points from results of the pegloticase trials include, first, the observation that pegloticase is a very potent ULT. Durable reductions in serum urate levels were observed in persistent responders (levels maintained below 6 mg per dL from starting treatment through to week 53), and 81% of persistent responders had complete or partial tophi resolution. Second, transient responders (patients who have a serum urate level higher than 6 mg per dL following treatment) can be identified by routine monitoring, usually within the first 3 months of therapy. Third, successful pegloticase therapy in patients with chronic gout refractory to conventional ULT (defined as serum urate levels <6 mg per dL 80% of the time in months 3 and 6 during the two Phase III trials), was associated with significant clinical benefit in tender joint and swollen joint counts and patient global assessment[10].

Fourth, infusion reactions were common and were the major reason for study withdrawal, whereas acute gout flares, owing to rapid lowering of serum urate levels, were the most common adverse events, being seen in 80% of patients. This reinforces the need for aggressive anti-inflammatory therapy to prevent further flares, which might include colchicine prophylaxis and/or corticosteroid therapy as used in the pegloticase trials, addition of interleukin-1β inhibitors, which have been shown to significantly reduce the risk of gout flares[11,12], or addition of methotrexate. Indeed, methotrexate in combination with infliximab (Remicade; Centocor Ortho Biotech) may prevent the formation of antibodies against infliximab and help maintain efficacy in patients with Crohn's disease[13], and it would be interesting to investigate whether methotrexate has such an effect when used with pegloticase. Anti-pegloticase antibodies, recognizing its PEG moiety, were detected in 89% of patients receiving pegloticase, and were associated with loss of treatment response, increased clearance of pegloticase and an increased risk of infusion reactions. So, it is recommended to discontinue pegloticase treatment in patients with serum urate levels >6 mg per dL, as well as in patients who have moderate to severe infusion reactions[8].

Overall, pegloticase provides a new option for patients with chronic gout who are refractory to conventional therapy. It might also be used in the future in patients who have a severe tophaceous burden and are seeking quicker tophi resolution — within months compared with years with oral ULT.

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Box 1 | The market for gout therapies

Analysing the market for gout therapies is Uma Yasothan, IMS Health, London, UK.

The global gout market is valued at ~US$900 million, with 50% of sales coming from outside the United States and Europe[14].The US and European markets have experienced significant growth rates in 2010, primarily driven by the launch of febuxostat (Uloric/Adenuric; Teijin Pharma/Takeda/Ipsen/Menarini), which recently became the first new drug to be approved for the treatment of gout in nearly four decades and had sales valued at $87 million in 2010 (REF. 14). For example, the gout market in the United States grew by more than 100% in 2010, from $100 million in 2009 (REF. 14). Sales of allopurinol, the cornerstone gout therapy, have remained relatively constant in recent years, suggesting increasing market potential for gout treatments, which could be due to factors including an increase in disease incidence, diagnosis or identification of allopurinol treatment failures.

In September 2010, the US FDA approved pegloticase (Krystexxa; Savient Pharmaceuticals) as the first drug for patients with gout who are refractory to conventional therapy. Given that pegloticase is expected to be used for a more focused target population than febuxostat, analyst expectations for sales range from $46 million to $53 million in 2010 to peak sales estimates of $313 million by 2015 (REFS 15,16).

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Competing financial interests
N.S. declares competing financial interests: see web version for details.

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U.Y. would like to thank D. Hanicq for assistance in data collection.

ANTICANCER DRUGS

Blocking phospholipid–protein interactions

Most strategies that target the phosphoinositide 3-kinase (PI3K) pathway — which is frequently dysregulated in tumorigenesis — have focused on inhibitors of downstream targets such as the protein kinase Akt. Now, Miao and colleagues describe a new way of modulating the pathway by blocking a key phospholipid–protein interaction, which suppresses tumour growth in a mouse cancer model.

The signalling molecule phosphatidylinositol-3,4,5-triphosphate ($PtdIns(3,4,5)P_3$; also known as PIP3) — which is the product of PI3K activity — controls cell growth, proliferation and survival through its interaction with proteins which contain pleckstrin homology (PH) domains. To identify inhibitors of this interaction, the authors performed a high-throughput screen of 50,000 small molecules, and found two compounds which disrupted the interaction between $PtdIns(3,4,5)P_3$ and the Akt PH binding domain with micromolar potency. Further characterization of one compound — termed PIT1 — suggested that it directly binds to the Akt PH domain, rather than targeting the lipid, and that the PIT1 binding site overlaps with the $PtdIns(3,4,5)P_3$ binding site.

The inhibitor exhibited selectivity towards a distinct subset of $PtdIns(3,4,5)P_3$-specific PH domains, including those of Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1), without affecting several $PtdIns(4,5)P_2$-selective PH domains. Comparison of the crystal structures of the PH domains of Akt, PDK1 and general receptor of phosphoinositides 1 (GRP1; a protein tyrosine kinase also known as cytohesin 3) showed that hydrophobic groups in the R2 and R3 positions of PIT1 would be preferred for Akt and disfavoured for PDK1 and GRP1 binding, suggesting that it would be possible to rationally modulate the selectivity of compounds that inhibited the interaction between $PtdIns(3,4,5)P_3$ and Akt PH binding domains.

> “Compounds such as PIT1 could be useful in the therapy of tumours characterized by elevated $PtdIns(3,4,5)P_3$ levels, such as glioblastomas.”

In glioblastoma cells — which show elevated basal levels of $PtdIns(3,4,5)P_3$ due to mutations in the phosphatase and tensin homolog (PTEN) — PIT1 suppressed PI3K-PDK1-Akt-dependent phosphorylation. Similar effects were also observed in growth factor-stimulated breast carcinoma cells. Moreover, the differential mechanism of action of PIT1 compared to several previously reported Akt inhibitors suggests that such compounds could be used in combination.

Next, the authors explored the potential anticancer activities of PIT1. The compound reduced the viability of multiple cancer cell lines, and caused preferential death of the cells which contained elevated $PtdIns(3,4,5)P_3$ levels (that is, cells with inactive PTEN) compared to cells expressing wild-type PTEN. PIT1 caused dysregulation of energy homeostasis, induced metabolic stress in cancer cells and led to the induction of autophagy. *In vivo*, a dimethyl analogue of PIT1 (used to improve solubility) attenuated tumour growth in a mouse model of breast cancer.

This study describes new small molecule antagonists of $PtdIns(3,4,5)P_3$–PH domain interactions, which could be used as leads for further optimization. Compounds such as PIT1 could be useful — either alone or in combination with existing Akt kinase inhibitors or PI3K inhibitors — in the therapy of tumours characterized by elevated $PtdIns(3,4,5)P_3$ levels, such as glioblastomas.

Charlotte Harrison

ORIGINAL RESEARCH PAPER Miao, B. *et al.* Small molecule inhibition of phosphatidylinositol-3,4,5-triphosphate (PIP3) binding to pleckstrin homology domains. *Proc. Natl Acad. Sci. USA* **107**, 20126–20131 (2010)

FURTHER READING Liu, P. *et al.* Targeting the phosphoinositide 3-kinase pathway in cancer. *Nature Rev. Drug Discov.* **8**, 627–644 (2009)

STROKE

Removing restraints on recovery

Recovery after stroke involves remapping of the neuronal circuitry in the regions adjacent to the site of injury — the peri-infarct zone — but so far there are no pharmacological therapies that can promote this. Now, Clarkson *et al.* show that inhibiting tonic GABA (γ-aminobutyric acid)-ergic signalling days after a stroke can improve locomotor function, suggesting a therapeutic approach that is less time sensitive than directly removing the obstruction to the cerebral blood vessel.

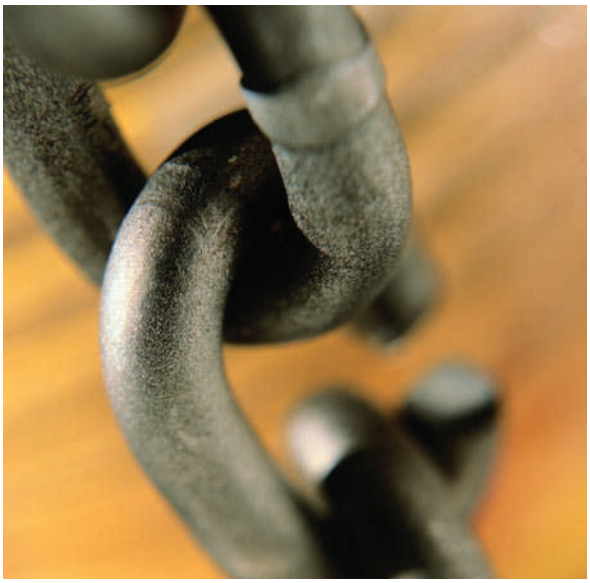
GABA signalling reduces neuronal excitability and thereby modulates synaptic plasticity. The authors proposed that this might prevent functional reorganization in the peri-infarct zone following stroke, and began by testing this hypothesis through electrophysiology studies in mouse brain slices. Whereas phasic GABAergic signalling was normal, tonic GABA transmission — which occurs in the absence of action potential firing and is mediated by extrasynaptic GABA type A receptors ($GABA_A$Rs) containing α5- or δ-subunits — in this region was increased in mice that had suffered a stroke compared with sham controls.

To investigate the underlying mechanisms, GABA transporters (GATs), which are responsible for GABA reuptake, were inhibited with GAT subtype-specific blockers. Together with western blot analysis, the results showed that reduced expression of GAT3 was responsible for increased GABAergic signalling after stroke.

What are the functional consequences of this increase in tonic GABAergic signalling after stroke? To address this question, the authors chronically treated mice with the inverse agonist L655,708, which antagonizes the α5-containing $GABA_A$R, beginning 3 days after stroke. Four days later, the mice showed improved forelimb and hindlimb motor control compared with vehicle-treated mice. Performance declined when treatment was discontinued after 2 weeks, but remained above that of controls.

To provide further support for the role of extrasynaptic GABA receptors in restraining recovery from stroke, the authors used transgenic mice lacking either α5- or δ-containing $GABA_A$Rs, and studied their performance in motor control tasks. In both cases, receptor knockout was associated with improved motor recovery after stroke, particularly in mice lacking the α5-containing $GABA_A$R.

Previous work has shown that reducing GABAergic signalling too soon after stroke can increase cell death, highlighting the importance of the timing of drug delivery. Consistent with these findings, mice treated with L655,708 at the onset of stroke had larger stroke volume than untreated mice and mice treated 3 days post-stroke, suggesting that delaying treatment may avoid exacerbating tissue damage.



> “Inhibiting tonic GABA-ergic signalling days after a stroke can improve locomotor function.”

Together, these findings suggest a pharmacological approach to re-establish functional neuronal connections that are lost during stroke, which could enhance current physical rehabilitation therapies and may have application in other brain injuries.

Katie Kingwell

ORIGINAL RESEARCH PAPER Clarkson, A. N. *et al.* Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature* **468**, 305–309 (2010)

ANALGESIA

Lipid linked to improved opiate therapy

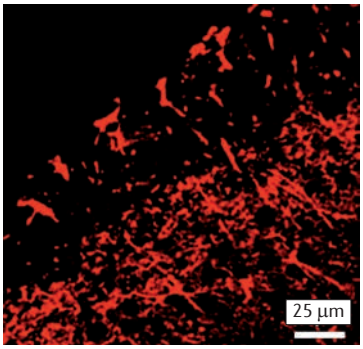


Image courtesy of D. Salvemini, Saint Louis University School of Medicine, Missouri, USA.

> Spinally formed sphingosine 1-phosphate (S1P) has a key role in opioid tolerance as well as opioid-induced hyperalgesia.

Opiates such as morphine are the most effective treatments for severe pain. However, patients often become tolerant to these drugs, requiring increasingly high doses for effectiveness, and this leads to dependence, over-sedation and other side effects. Now, Salvemini and colleagues have shown that spinally formed sphingosine 1-phosphate (S1P) has a key role in opioid tolerance as well as opioid-induced hyperalgesia (hypersensitivity to pain) and that inhibitors of the production and activity of S1P could have the potential to address these limitations of opioid therapy. Furthermore, as reported in *The Journal of Neuroscience*, they find that S1P acts in two ways on spinal glial cells: it causes both inflammatory cytokine release and post-translational nitration of enzymes thought to be involved in sensitization to pain.

S1P is produced from ceramide by the sphingosine kinases SPK1 and SPK2 and is the end product of the ceramide metabolic pathway. S1P acts on cells throughout the body, including neurons and glia in the central nervous system. Several S1P inhibitors are known to have anti-inflammatory effects — one example is fingolimod (Gilenya; Novartis), which was recently approved for the treatment of multiple sclerosis. From their previous studies, the authors knew that ceramide biosynthesis is associated with morphine tolerance, but it was not known whether the ceramide–S1P pathway is involved. To test this in rats, the authors co-administered morphine and a sphingosine kinase inhibitor (either DMS or SK-I) and found that morphine-induced tolerance and hyperalgesia — as assessed by the time taken to respond to painful stimuli — were significantly reduced.

The authors also found that the rats that developed tolerance and hyperalgesia following morphine administration had higher levels of S1P, as measured in spinal extracts of sphingolipids. Spinal ceramide, measured by immunofluorescence, was similarly elevated in these animals and colocalized with markers for two kinds of glia (microglia and astrocytes) but not with markers for neurons. DMS prevented these effects and also prevented a morphine-induced increase in the inflammatory cytokines tumour necrosis factor, interleukin-1β and interleukin-6, which are thought to be involved in neuronal excitability.

In addition to these anti-inflammatory effects, DMS attenuated a morphine-induced increase in peroxynitrite in the rats' spines. Peroxynitrite can nitrate two glial cell proteins thought to have a role in the sensing of pain — GLT1 and glutamine synthetase (GS) — deactivating them as a result. Their deactivation is thought to enhance glutamate neurotransmission, which is important in central sensitization and might therefore play a part in the development of hyperalgesia. The morphine-induced nitration of these proteins was reduced by DMS, establishing a link between S1P and the peroxynitrite-mediated nitration of GLT1 and GS.

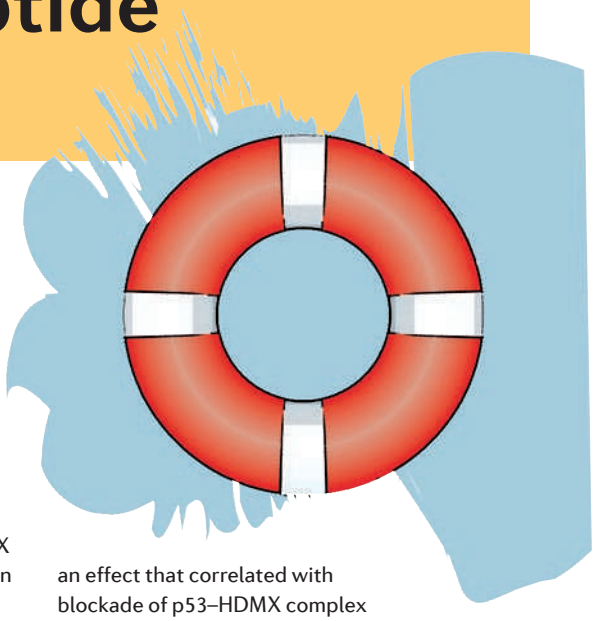
This study is significant because of the large number of patients who could potentially benefit from improved pain management with opiates by co-administering agents that target S1P production or its effects. However, precisely how morphine boosts ceramide levels and how S1P modulates inflammatory cytokines and causes protein nitration remain to be discovered.

Samia Burridge

ORIGINAL RESEARCH PAPER Muscoli, C. *et al.* Counter-regulation of opioid analgesia by glial-derived bioactive sphingolipids. *J. Neurosci.* **30**, 15400–15408 (2010)
FURTHER READING Brinkmann, V. *et al.* Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nature Rev. Drug Discov.* **9**, 883– 897 (2010) | Woolf, C. J. Overcoming obstacles to developing new analgesics. *Nature Med.* **16**, 1241–1247 (2010)

ANTICANCER DRUGS

Stapled peptide rescues p53



Loss of the tumour-suppressing activity of the p53 pathway occurs in many cancers, so targeting the negative regulators — HDM2 (also known as MDM2) and HDMX (also known as MDM4), which are overexpressed in some tumours — has been pursued as an anticancer strategy. Now, Walensky and colleagues explore the therapeutic potential of a 'stapled' p53-based peptide that prevents p53–HDMX binding, enabling activation of the p53 response and tumour growth suppression. A strategy for determining which cancers HDMX and/or HDM2 inhibition may be most effective in is also revealed.

Previously, peptides and small molecule HDM2 inhibitors (such as nutlin 3) have been developed that block p53–HDM2 binding, triggering cell cycle arrest and apoptosis in cancer models. However, overexpression of HDMX, which binds to and sequesters the HDM2 inhibitor-mediated elevated p53, renders some cancer cells resistant to this approach. Selectively targeting HDMX has therefore become an attractive alternative strategy. Here, Walensky and colleagues investigate the ability of peptides based on the transactivation domain of p53 to inhibit HDMX activity and exert tumour suppressing activity. These peptides are 'stapled' by installation of a hydrocarbon linkage to restore the α-helical structure, confer protease resistance and promote cellular uptake.

One such stabilized α-helix (SAH) of p53, SAH-p53-8, was found to have a 25-fold greater binding preference for HDMX over HDM2. This peptide demonstrated dose-dependent cytotoxicity in cancer cell lines overexpressing HDM2, HDMX or both proteins. Strikingly, this stapled peptide was most effective in cells expressing high HDMX and p53 levels, which were most resistant to nutlin 3. Mechanistic studies confirmed that SAH-p53-8 acted by blocking HDMX-mediated p53 sequestration, thereby restoring the p53 pathway.

Next, a series of synergy experiments in various cancer cell lines revealed in which cellular context HDMX and/or HDM2 inhibition would be most effective. In MCF7 cells that develop resistance to nutlin 3 owing to high HDMX levels, SAH-p53-8 restored nutlin 3 activity. Moreover, combining the two agents enhanced cytotoxicity, an effect that correlated with blockade of p53–HDMX complex formation. By contrast, in JEG3 cells, nutlin 3 did not synergize with SAH-p53-8 as endogenous p53 levels are already elevated in these cells. And in SJSA1 cells, which exhibit low HDMX and p53 expression levels but high HDM2 expression, SAH-p53-8 provided no added benefit over nutlin 3. However, enforcement of HDMX expression in SJSA1 cells engendered nutlin 3 resistance and restored the synergistic benefit of co-treatment.

Together, these *in vitro* studies indicate that targeting HDMX will be most effective in tumours in which p53 levels are endogenously or pharmacologically elevated. Moreover, the presence of the p53–HDMX complex may represent a potential biomarker to predict therapeutic efficacy.

Finally, to further demonstrate the therapeutic potential of SAH-p53-8, the authors investigated the *in vivo* actions of this stapled peptide, using a JEG3 mouse xenograft model — an HDMX-expressing and HDM2 inhibitor-resistant cancer. Four days of intravenous SAH-p53-8 treatment reduced tumour burden by 37–46% compared to control mice and nutlin 3 mice. Importantly, p53 pathway reactivation was confirmed and there were no signs of toxicity.

This study provides further confirmation that the p53 antagonists, HDM2 and HDMX, are viable anticancer targets. Clinical validation of this approach is needed.

Sarah Crunkhorn

ORIGINAL RESEARCH PAPER Bernal, F. *et al.* A stapled p53 helix overcomes HDMX-mediated suppression of p53. *Cancer Cell* **18**, 411–422 (2010)

METABOLIC DISEASE

GOAT inhibitors to battle the bulge?

Much research in the quest to develop a safe and highly effective obesity treatment has focused on deciphering the nutrient–hormone interactions that influence weight gain. The gastric peptide hormone ghrelin has been identified as a central player in this process, but strategies to target its active form, acyl ghrelin, have had limited success. Now, reporting in *Science*, Barnett and colleagues present a peptide inhibitor of ghrelin-O-acetyltransferase (GOAT) that blocks the formation of acyl ghrelin and reduces weight gain in mice fed a high-fat diet.

The inhibitor, GO-CoA-Tat, was designed to mimic and 'lock' a putative ternary complex formed between GOAT and its substrates octanoyl-CoA and ghrelin. To achieve this, amino acids 1–10 of ghrelin were linked with a non-cleavable bond to octanoyl-CoA and coupled to an 11-mer HIV Tat-derived sequence to promote membrane permeation.

GO-CoA-Tat specifically and directly interacted with GOAT in cell lines expressing GOAT and pro-ghrelin, and reduced acyl ghrelin formation. Intraperitoneal injection of GO-CoA-Tat in mice fed a high-fat diet resulted in a relative reduction of fat mass, but no difference in lean mass, compared with vehicle-treated or ghrelin-knockout mice. Serum levels of acyl ghrelin, glucose and insulin-like growth factor 1 were reduced, whereas the level of desacyl ghrelin remained unchanged.

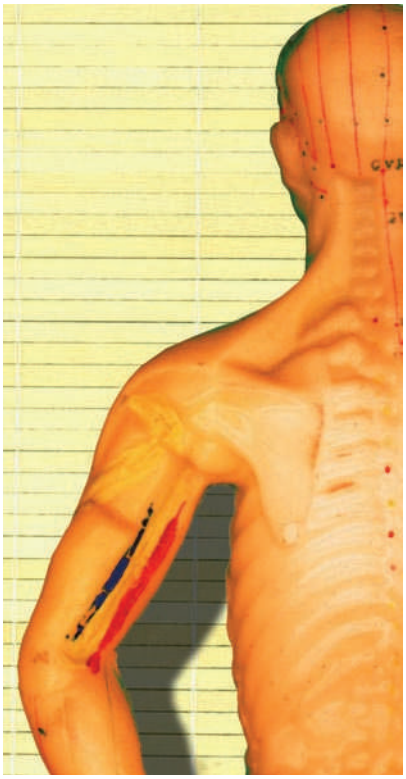
To decipher the effect of GOAT inhibition on glucose homeostasis and insulin levels, isolated human pancreatic islet cells were challenged with glucose. Cells that were pretreated with GO-CoA-Tat had a significantly higher insulin response compared with untreated cells. *In vivo*, GO-CoA-Tat pretreatment of glucose-challenged wild-type mice, but not ghrelin-knockout mice, resulted in significantly higher serum insulin levels and a reduction in blood glucose. A small population of ghrelin-expressing cells were identified in pancreatic islets. Further analysis demonstrated a 20-fold reduction of the mRNA coding for uncoupling protein 2 (UCP2), a suppressor of insulin secretion, in pancreatic islet cells from mice treated with GO-CoA-Tat. These data point to a tissue-specific role for GOAT inhibition in suppressing UCP2 levels and in enhancing insulin release in response to glucose, providing a further link between acyl ghrelin, obesity and type 2 diabetes.

The authors point out that targeting the biosynthesis of acyl ghrelin can have several advantages over approaches to block its receptor. As acyl ghrelin formation can be blocked in the periphery, the drug does not need to cross the blood–brain barrier. Also, targeting an enzyme may be easier than targeting an abundant receptor. Furthermore, there has been concern that acyl ghrelin receptor antagonists can increase the level of acyl ghrelin. While peptide inhibitors have obvious limitations, synthetic derivatives of GOAT inhibitors like GO-CoA-Tat could be developed into a promising tool for the management of metabolic disorders.

Alexandra Flemming

ORIGINAL RESEARCH PAPER Barnett, B. P. *et al.* Glucose and weight control in mice with a designed ghrelin O-acetyltransferase inhibitor. *Science* 18 Nov 2010 (doi:10.1126/science.1196154)

IN BRIEF



DRUG DELIVERY

Rapid translocation of nanoparticles from the lung airspaces to the body

Choi, H. S. *et al. Nature Biotech.* **28**, 1300–1303 (2010)

This study investigated the movement of nanoparticles from the lungs into lymph nodes and the bloodstream, and subsequent elimination of these nanoparticles from the body. They found that making nanoparticle-based drugs zwitterionic and <6 nm in diameter should result in rapid and high levels of drug accumulation in the bloodstream while permitting renal clearance of drug that is not bound to its target. Non-cationic nanoparticles with a diameter of ≤34 nm and ≥6 nm deliver high levels of a drug to pulmonary lymph nodes, which could be used to deliver antibiotics and anti-inflammatory drugs.

G PROTEIN-COUPLED RECEPTORS

Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist

Chen, E. Y. T. *et al. Science* **330**, 1091–1095 (2010)

Homology between the dopamine D3 receptor (D3R) and the D2R makes the identification of D2R-selective ligands challenging. This study determined the crystal structure of the human D3R, revealing novel features of the ligand-binding pocket. For a ligand to be selective for the D3R, in addition to binding to a site that is common to D2R and D3R, it must extend towards an extracellular part of the binding pocket. This identification of an extracellular binding site that differs between D2R and D3R could aid the design of D3R-selective agents for treating drug abuse and other neuropsychiatric indications.

CARDIOVASCULAR DISEASE

Exogenously administered secreted frizzled related protein 2 (Sfrp2) reduces fibrosis and improves cardiac function in a rat model of myocardial infarction

He, W. *et al. Proc. Natl Acad. Sci. USA* **107**, 21110–21115 (2010)

Bone morphogenetic protein 1 (BMP1)–Tolloid-like metalloproteinases are involved in collagen maturation and deposition, so they are targets for the modulation of fibrosis. This paper shows that secreted frizzled-related protein 2 (SFRP2) inhibits BMP1 activity and has beneficial effects in cellular models of fibrosis and animal models of cardiac dysfunction. Injection of SFRP2 into rat infarcted myocardium inhibited type I collagen deposition, reduced left ventricular fibrosis, prevented anterior wall thinning and improved cardiac function.

SCREENING

Superfamily-wide portrait of serine hydrolase inhibition achieved by library-versus-library screening

Bachovchina, D. A. *et al. Proc. Natl Acad. Sci. USA* **107**, 20941–20946 (2010)

There is a lack of biological assays and inhibitors with which to study the serine hydrolase family of metabolic enzymes. Here, activity-based protein profiling — a technique that uses site-directed chemical probes to covalently label large numbers of related enzymes — was used to identify lead carbamate-based inhibitors for over 30 serine hydrolases, including several uncharacterized enzymes. This approach may potentially be applied to other enzyme families for which activity-based probes have been developed.

OUTLOOK

Competitiveness in follow-on drug R&D: a race or imitation?

Joseph A. DiMasi and Laura B. Faden

Abstract | The development of 'follow on' or 'me too' drugs — generally defined as a drug with a similar chemical structure or the same mechanism of action as a drug that is already marketed — has attracted contrasting views. Some have argued that follow-on drugs often provide useful alternative or enhanced therapeutic options for particular patients or patient subpopulations, as well as introducing price competition. Others, however, consider that the development of such drugs is duplicative and that the resources needed would be better directed elsewhere. Implicit in some of this criticism is the notion that the development of me-too drugs is undertaken after a first-in-class drug has made it to market and proved commercially successful. In this Perspective, using analysis of development and patent filing histories of entrants to new drug classes in the past five decades, we provide new evidence that the development of multiple new drugs in a given class is better characterized as a race, rather than the imitation of successful products.

The development of first-in-class drugs, such as those with a new mechanism of action, is widely considered to reflect genuine and valuable innovation. Conversely, the development and the marketing of further new drugs in a given therapeutic class — often described as 'me too' drugs — has been criticized by some as duplicative and wasteful, who argue that the resources required should be used instead to develop more innovative products[1,2]. However, it has also been argued that companies do not set out to develop drugs that have no added value and that additional drugs in a given class provide value by both expanding options for individual patients or patient subgroups and by creating some degree of price competition (see REFS 3–7 for further discussions of the clinical and economic benefits of such products).

With the aim of providing insights into the nature of the research and development (R&D) activities leading to the development of multiple entrants in a therapeutic class, which we characterize using the more value-neutral terminology of 'follow on' drug[6], we analyse the timing of patent filing and the entry of products in various drug classes to the United States market over the past 50 years (see BOX 1 for details). This study updates and extends our previous analysis of the competitiveness of pharmaceutical R&D[8], published in 2004, which found that the period of marketing exclusivity for the first drug in a new class fell substantially from a median of 10.2 years in the 1970s to 1.2 years in the late 1990s. For the current analysis, we expanded the number of drug classes and time periods studied to include more recent data and included patent data for the first time.

Our previous study noted that the literature on speed to entry in pharmaceutical markets at that time was not extensive[9,10] and since then we note one other addition to the literature[11]. This study did examine the lag between first and second entrants in drug classes, defined by a single formulary, but the emphasis in the paper was on whether first-in-class drugs with no competition in a highly specific class, or long lags between approvals for first-in-class and second entrants in the class, pose a considerable financial problem for the US Medicare health insurance programme (given limited price competition). Although the set of drug classes differed to some extent, this study confirmed our previous study results on the substantial increase in the speed of competitor entry in drug classes over time.

This current study is based on an extensive list of new drug classes that received marketing approval in the United States between the 1960s and the early 2000s (BOX 1). To allow for a reasonable amount of time for the entry of subsequent drugs in a new class to occur, we restricted the search for first-in-class compounds to new drugs approved up to 2003 and examined the follow-on new drug approvals in each class up to 2007. For the drug classes identified, we examined the therapeutic significance ratings that the US Food and Drug Administration (FDA) assigned to new follow-on drugs at the time of marketing approval. Finally, we investigated the development and patent filing histories of follow-on drugs in a drug class and compared them to the approval dates and the development and patent filing histories of their corresponding first-in-class drugs.

Characteristics of drug classes

We identified 97 drug classes in which the first-in-class compound was approved in the United States between 1960 and 2003, and 94 of these classes were analysed; three classes in which the same sponsor developed all of the entrants were excluded. In these 94 therapeutic classes, 287 follow-on drugs were approved between 1960 and 2007 and so the mean number of compounds per class is 4.1 (including the first-in-class compound). The number of drugs per class ranged from 2–14, with a median of three, and 72% of the classes have four or fewer compounds in them.

The extent to which there is entry in a class does not differ according to the therapeutic review rating of the first-in-class drug; the number of drugs per class is 4.1 for both priority-rated and standard-rated first-in-class categories (although therapeutic ratings were not available for eight of the first-in-class drugs; seven were biologics not given ratings by their reviewing division and one was a small molecule too old to have a rating).

Box 1 | **Data sources and analysis**

The Tufts Center for the Study of Drug Development (Tufts CSDD) maintains databases of new drugs and biopharmaceuticals approved in the United States. We used these databases to provide a list of new chemical entities and new biopharmaceuticals approved in the United States from 1960 onwards (here, we refer to both types of compounds as new drugs). New diagnostic drugs and new salts, esters or formulations of existing drugs were excluded from the analysis.

As in our previous study[8], a therapeutic class was defined to consist of new drugs that had a similar chemical structure or the same pharmacological mode of action and that were used primarily for the same indications. We established drug classes and investigated development histories using information from a wide range of sources, including Tufts CSDD databases, commercial databases (The Investigational Drugs Database (IDdb3), *The NDA Pipeline*, PharmaProjects and IMS R&D Focus), the Physicians' Desk References, the Medical Letter, the Merck Index, the US Food and Drug Administration (FDA), clinical pharmacology websites, pharmacopeias (United States Pharmacopeia Dispensing Information and American Hospital Formulary Service) and the United States Pharmacopeia Model Formulary developed in association with the Medicare Part D drug benefit. We obtained patent information on first-in-class and follow-on drugs from the IMS Patent Focus database.

From an economic perspective, our definition of a drug class is conservative, as drugs in one class will often compete to some extent with drugs from other classes that are used to treat the same conditions. For example, numerous drug classes are available for the treatment of hypertension (although drugs from different classes are often given in combination), histamine receptor type 2 antagonists and proton-pump inhibitors have competed in gastrointestinal markets, and drugs from varied classes are used to control pain. Our focus, however, is on the rate of entry and the rate of development of 'me too' drugs (a term that can be traced back to at least the 1960s[15]), which has historically referred to a new drug entity that is a new entrant to a therapeutic class, defined by a separate drug entity that was the first in the class to obtain marketing approval.

To allow for a reasonable amount of time for entry of competitors in a given drug class to occur, we restricted the search for first-in-class compounds to new drugs approved up to 2003. The approvals of follow-on new drugs in each class that we examined were approved up to 2007. To analyse trends in the entry of competitors, the data were partitioned into the following periods: 1960–1969; 1970–1979; 1980–1984; 1985–1989; 1990–1994; 1994–1999 and 2000–2003.

Given that the period of data collection is necessarily restricted in time, there is inevitably a potential that relevant future data on follow-on drugs in the 94 classes analysed has not been included. However, we consider that this is not a significant concern for our analysis of the timing of the initial entry of competitors in a given class for the following reasons. Effective patent lifetimes for new drugs have averaged around 11–12 years and competition from generic versions is intense once it occurs[16]. In addition, new, often improved, drug classes frequently arise to treat the same conditions. For first-in-class drugs approved in the most recent period analysed (2000–2003), it took 4–8 years for follow-on entry to occur and for those approved in the next most recent period (1995–1999) it took 8–13 years. So, any loss of potential data on follow-on drugs is only likely to be important for the most recent period. Even in this case, given the expense of new drug development[17], the incentives for firms to pursue and obtain new drug approvals in the future in those classes for which follow-on drugs have not already been approved are relatively low.

As in our previous study[8], for some of our analyses, the new drugs were grouped according to the FDA's therapeutic rating system for new drug approvals. In late 1975, the FDA established a three-tiered rating system for prioritizing the review of new drug applications, with new drugs thought at the time to represent a significant gain over existing therapy, a modest gain over existing therapy and little or no gain over existing therapy, being given an A, B and C rating, respectively. In addition, the FDA retroactively rated new drugs approved during 1963 to 1975 in accordance with this scheme. In 1992, the FDA altered its rating system to contain two tiers: priority (P) or standard (S). So, for the purposes of this analysis, which spans both periods, we grouped all the new drugs into two categories: 'priority rated', new drugs that had received an A or B rating, or a P rating; and 'standard rated', new drugs that had been assigned a C rating or an S rating.

Speed of entry of follow-on drugs

To investigate trends in the speed at which follow-on competitors entered the market in a given drug class, we divided the data into periods based on when the first-in-class drug was approved for marketing in the United States, as described in BOX 1. The 94 drug classes, the first-in-class drug, the first follow-on drug in the class to be approved and the period of class marketing exclusivity for the original drug are provided in Supplementary information S1 (table). Data were grouped on the basis of the period during which the first-in-class drug was approved and the means and the medians for those periods were calculated.

As shown in FIG. 1a, the mean length of the period of marketing exclusivity for the first entrants in a drug class has declined substantially since the 1970s, falling 82% from the 1970s to 1995–1999 (9.7 to 1.7 years). The time from first-in-class approval to first follow-on approval in a class was also regressed linearly on the year in which the first-in-class drug was approved, and this analysis is presented in TABLE 1. The period of analysis was allowed to vary by including or excluding the most recent period in FIG. 1 and by including or excluding the 1960s, as the data in FIG. 1a do not suggest a decline in the speed of entry from the 1960s to the 1970s. The regressions suggest that the speed at which the market entry of competitors to first-in-class drugs occurred increased at the rate of approximately 3 years per decade. Looking beyond the first follow-on entrant to a given drug class, the mean and median times to second and third follow-on entrants in a class (TABLE 2) also indicate that competition increased over time because later entrants tended to enter the market sooner.

Is the first drug in a class the best?

As highlighted above and discussed in our previous study[8], the first drug to be approved in a given class is often considered as the breakthrough product, whereas the clinical value of subsequent entrants to the class has been questioned. It is beyond the scope of this article to extensively analyse the clinical properties of the drugs in our data set but some insights into the clinical value of follow-on drugs can be gained from examining the therapeutic ratings that the FDA has assigned to follow-on drugs. The FDA has used such rating systems to help prioritize the allocation of regulatory resources to the review of new drug applications. As described in BOX 1, we have used the FDA classifications of new drugs to group them into two categories: 'priority rated' drugs, which are generally considered to offer significant advances in treatment or to provide a treatment for a condition for which no adequate therapy already exists, and 'standard rated' drugs, which are considered to offer only minor improvements, at most, over existing treatments.

With this in mind, in the case of follow-on drugs, it seems plausible that the FDA would be unlikely to give a priority rating for a follow-on drug in the same class for what might be fairly modest improvements in efficacy, safety or convenience compared with the first-in-class drug. Nonetheless, as in our earlier study[8], our data show that nearly one-third of all follow-on drugs have received a priority rating from the FDA (32%) and that 57% of all classes have at least one follow-on drug that received a priority

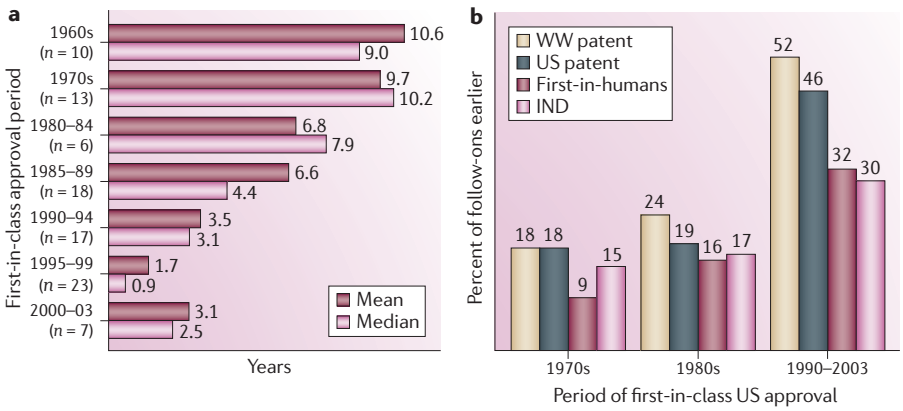


Figure 1 | **Speed of entry and development of follow-on drugs. a** | The graph shows the average period of marketing exclusivity for first entrants to a therapeutic class (time from first-in-class approval to first follow-on drug approval) by period of first-in-class United States marketing approval. Analysis of variance results indicate that the differences in means across periods are highly statistically significant ($F_{6,87} = 5.29$, $P < 0.0001$). In addition, without making the normality assumptions necessary for an analysis of variance test, the non-parametric Kruskal–Wallis test indicates that the medians are different by statistically significant amounts ($\chi^2(6) = 30.05$, $P < 0.0001$). **b** | The graph shows the percentage of follow-on drugs approved in the United States from 1970 to 2007 that had a first patent filed anywhere in the world (WW patent), or had a first patent filed in the United States (US patent), or were first tested in humans anywhere in the world (first-in-humans) or had an investigational new drug (IND) filed, before the first-in-class compound (for therapeutic classes in which the first-in-class drug was approved in the United States from 1970 to 2003). The IND results for the 1960s may be skewed as the IND process was not initiated in the United States until 1963, and INDs were filed in 1963 for drugs that had already been in clinical testing in the United States in previous years. Otherwise, however, the results are conservative because we do not have complete information on the development histories of the drugs.

rating. Furthermore, given that it is unlikely that relatively minor improvements in an existing drug class will result in a priority rating from the FDA, it is likely that these values are an underestimation of the extent to which the best-in-class drug is not the first-in-class drug.

A substantial number of later follow-on drugs had priority ratings, as also noted in the previous study[8]. More than one in five of the follow-on drugs with priority ratings (19 out of 90) were the fourth or later follow-on drug to be approved. About 45% of the classes had four or more follow-on drugs and in this set of classes, 44% of the follow-on drugs that had received a priority rating (19 out of 43) were the fourth or later follow-on.

Imitation or development races?

Implicit in some of the criticism of the development of me-too drugs has been the assumption that their development occurs following the demonstration of clinical and commercial success by the first-in-class drug. However, given assessments of the length of time that is typically required for drug development — estimated at between 10 to 15 years[12] — the data on the timing of entry of follow-on drugs in a particular class, in this study and in our previous study, suggest that much of the development of what turn out to be follow-on drugs must occur before the approval of the breakthrough drug.

To investigate this issue for various phases of drug development, as in our earlier study[8], we have used the Tufts Center for the Study of Drug Development data (BOX 1) on milestones in the development process of the drugs in the classes analysed. For all drug classes introduced since the late 1980s, at least one follow-on drug was synthesized before the approval of the first-in-class drug and the first pharmacological testing of some follow-on drugs occurred before the first-in-class drug was approved, in all but one class, since the late 1980s. Indeed, initial clinical testing of at least one follow-on drug in a class occurred before the approval of the first-in-class drug for at least 80% of the classes in each of the periods since the late 1980s. At least half of all classes had at least one follow-on drug for which the investigational new drug application (IND) was filed before the approval of the first-in-class drug for each period since the early 1980s, with this being the case for 89% of the classes since the early 1990s. At least half of the classes in any period since the early 1980s had at least one follow-on drug with Phase II testing initiated before the first drug in the class was approved. Since the late 1980s, 64% of the classes had at least one follow-on drug in the class with Phase III testing initiated before first-in-class approval, with the share at 88% since the late 1990s.

To help illustrate this issue, TABLE 3 shows the proportion of follow-on drugs that had reached various development milestones before the first-in-class drug was approved. All of the follow-on drugs for classes in which the first-in-class drug was approved from the late 1980s onwards were synthesized before the first-in-class drug was approved. Indeed, for classes in which the first member was approved since 1990, 80% or more of the follow-on drugs were in clinical trials somewhere in the world before the approval of the first-in-class drug. Moreover, Phase II trials had begun for more than 70% and Phase III trials had begun for more than 60% of the follow-on drugs in classes in which the first-in-class drug was approved from the late 1990s onwards.

To further investigate the nature of the development of follow-on drugs, we have

Table 1 | **Regression results for trends in the time to market entry following a first-in-class approval**

Period of first-in-class approval	Intercept*	*P* value	YEARONE*‡	*P* value	R^2	Estimated decline in time to entry per decade
1960–1999	578.1716 (113.6448)	<0.0001	–0.2883 (0.0572)	<0.0001	0.23	2.9 years
1960–2003	544.7796 (102.0714)	<0.0001	–0.2714 (0.0514)	<0.0001	0.22	2.7 years
1970–1999	676.4947 (128.4984)	<0.0001	–0.33377 (0.0646)	<0.0001	0.27	3.4 years
1970–2003	608.4824 (112.6502)	<0.0001	–0.3034 (0.0566)	<0.0001	0.26	3.0 years

*The coefficient estimates are given with their standard error in parentheses. ‡The YEARONE variable represents the year in which a first-in-class drug was approved for marketing in the United States.

Table 2 | **Average time to market entry for second and third follow-on drugs**

Period of US marketing approval for first entrant in class	Time from first to second follow-on drug (years)			Time from second to third follow-on drug (years)		
	Mean	Median	*N*	Mean	Median	*N*
1960s	13.5	16.1	9	8.3	5.1	5
1970s	5.7	4.2	12	5.2	3.7	8
1980s	3.8	3.5	18	2.8	2.0	15
1990s	2.7	2.0	28	2.2	1.3	14
2000–2003	1.1	1.1	4	NA	NA	NA

NA, not available. Analysis of variance results for testing whether there are differences in means across the time periods for the time to a second follow-on entrant ($F_{6,64} = 10.20$, $P < 0.0001$) and for the time to a third follow-on entrant ($F_{5,36} = 2.72$, $P = 0.0349$) showed highly statistically significant differences. Similarly, the Kruskal–Wallis test results are highly significant for both the time to a second follow-on entrant ($\chi^2(6) = 27.20$, $P < 0.0001$) and the time to a third follow-on entrant ($\chi^2(5) = 14.94$, $P = 0.0106$).

also examined the timing of the filing of the first United States and the first worldwide patents for follow-on drugs in relation to when the first-in-class drugs in their respective classes were approved, given that such patents are often first filed before the start of clinical testing. For drug classes in which the first-in-class drug had been approved since the 1970s, 90% had at least one follow-on drug in its class with its first worldwide patent filed before the first-in-class drug was approved, and 89% had at least one follow-on drug from the class with its first US patent filed before the first-in-class drug was approved. For classes in which the first-in-class drug had been approved from the early 1990s onwards, 98% had a follow-on drug in its class with a first worldwide patent or a first US patent approved before the first-in-class drug was approved.

The data on the shares of follow-on drugs with first patent filings before first-in-class approval are more comprehensive than for the data on development milestones. The results for patent filings, however, are consistent with, if not more striking than, those for development milestones. For the first-in-class approval periods that we examined since the early 1990s, 91% of the follow-on drugs had US patents filed before the first drug in their class was approved in the United States.

Overall, these results indicate that new drug development is better characterized as a race to market among drugs in a new therapeutic class, rather than a lower risk imitation of a proven breakthrough. This conclusion is further supported by the comparison of the development history of the first-in-class drug with those of the follow-on drugs in its class. As shown in FIG. 1b, nearly one in three follow-on drugs from classes in which the first drug was approved from 1990 to 2003 entered clinical testing either in the United States or elsewhere in the world before the first-in-class drug did. In addition, approximately one-half of the follow-on drugs from this period had a US or a worldwide patent filed before the corresponding first patent filing for the first-in-class drug. Thus, in terms of the timing of patent filing, the first drug in the class to be approved is, in aggregate, only approximately the median drug for the class.

R&D competitiveness and innovation

Our analysis of the timing of market entry of follow-on drugs in particular classes indicates that the length of time during which the first-in-class drug does not experience direct competition from a follow-on drug has generally diminished substantially over time. Although the most recent data (2000 to 2003 approvals for first-in-class drugs) shows somewhat lengthier times to approval for a second entrant into the drug class relative to the late 1990s period, the speed of entry of follow-on drugs is still more rapid than in the earlier periods analysed.

As discussed in detail in our earlier paper[8], there are various factors on both the supply and the demand sides of pharmaceutical markets that may have contributed to the increased competitiveness in drug classes. These include increased opportunities from advances in biomedical science; shifts in drug development approaches that make connectedness to scientific networks more important; legislation making generic entry easier; greater price sensitivity from managed care and foreign reimbursement authorities; and expansion of prescription drug insurance to a larger segment of the population.

Overall, the data that we examined on development and on patent filing histories

Table 3 | **Share of follow-on drugs with development phase initiated prior to first-in-class approval in various periods**

Development Phase	Percent initiated prior to first-in-class approval						
	1960s	1970s	1980–1984	1985–1989	1990–1994	1995–1999	2000–2003
Synthesis	37% (*n* = 27)	47% (*n* = 26)	71% (*n* = 17)	100% (*n* = 16)	100% (*n* = 17)	100% (*n* = 15)	100% (*n* = 3)
First pharmacological test	31% (*n* = 26)	41% (*n* = 27)	69% (*n* = 16)	71% (*n* = 21)	86% (*n* = 22)	94% (*n* = 17)	100% (*n* = 2)
First worldwide patent	26% (*n* = 31)	42% (*n* = 43)	62% (*n* = 29)	71% (*n* = 63)	91% (*n* = 44)	96% (*n* = 50)	89% (*n* = 9)
First United States patent	21% (*n* = 28)	35% (*n* = 43)	62% (*n* = 29)	71% (*n* = 63)	88% (*n* = 43)	94% (*n* = 50)	89% (*n* = 9)
First in humans anywhere	20% (*n* = 35)	32% (*n* = 34)	43% (*n* = 21)	65% (*n* = 34)	82% (*n* = 28)	93% (*n* = 27)	80% (*n* = 5)
Investigational new drug filing	19% (*n* = 37)	16% (*n* = 44)	26% (*n* = 31)	45% (*n* = 64)	49% (*n* = 45)	78% (*n* = 50)	70% (*n* = 10)
Phase II trial	4% (*n* = 29)	11% (*n* = 28)	26% (*n* = 19)	47% (*n* = 38)	68% (*n* = 19)	70% (*n* = 23)	75% (*n* = 4)
Phase III trial	4% (*n* = 29)	13% (*n* = 31)	10% (*n* = 21)	38% (*n* = 39)	36% (*n* = 28)	62% (*n* = 26)	100% (*n* = 3)

for first-in-class and follow-on drugs demonstrate that new drug development in drug classes has largely been done simultaneously in the more recent periods. Since the early 1990s, the overwhelming share of follow-on drugs that have been approved for marketing had been developed at least to the point of clinical testing before the first-in-class drug had been approved, and high percentages of follow-on drugs from this period were in late-stage clinical testing or in regulatory review before the first-in-class drug was approved. Indeed, since the early 1990s, nearly one in three follow-on drugs had entered clinical testing before the first drug in the class to be approved had done so. The results on patent filings for classes from this period are even more striking. Approximately one-half of what turned out to be follow-on drugs had a US or a worldwide patent filed before the first-in-class drug had any such patents filed.

Drug development can therefore often be characterized as a race in which several firms pursue investigational drugs with similar chemical structures or with the same mechanism of action before any drug in the class obtains regulatory marketing approval. So, the distinctions that are often drawn between the relative innovative value of the development of the first-in-class and the me-too drugs in the same class may be misguided. Aside from a potential impact on pricing, because different drugs in a class may have different effects on individuals, this competition in R&D can have the positive effect of expanding the number of patients who can be effectively and safely treated. Overall, this has important implications for optimizing the allocation of resources in pharmaceutical R&D. As is the case for other forms of research, innovation may be best served by more or less simultaneous independent development of numerous approaches — in this case, investigational drugs — to solving problems[13,14].

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets

Lynn Bedford, James Lowe‡, Lawrence R. Dick§, R. John Mayer* and James E. Brownell§*

Abstract | The ubiquitin–proteasome system (UPS) and ubiquitin-like protein (UBL) conjugation pathways are integral to cellular protein homeostasis. The growing recognition of the fundamental importance of these pathways to normal cell function and in disease has prompted an in-depth search for small-molecule inhibitors that selectively block the function of these pathways. However, our limited understanding of the molecular mechanisms and biological consequences of UBL conjugation is a significant hurdle to identifying drug-like inhibitors of enzyme targets within these pathways. Here, we highlight recent advances in understanding the role of some of these enzymes and how these new insights may be the key to developing novel therapeutics for diseases including immuno-inflammatory disorders, cancer, infectious diseases, cardiovascular disease and neurodegenerative disorders.

Ubiquitin
A highly conserved 76 amino-acid protein that can be reversibly attached to other proteins. Key structural features of ubiquitin include its β-grasp fold (a characteristic of all ubiquitin-like proteins), its C-terminal tail and seven lysine residues through which polyubiquitin chains are linked.

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Protein homeostasis is essential for most cellular processes. The ubiquitin–proteasome system (UPS) is responsible for much of the regulated proteolysis in the cell, and has non-degradative functions as well. Ubiquitin is a small 76 amino-acid protein that can be reversibly attached to other proteins and lies at the core of an elaborate post-translational modification pathway. Several ubiquitin-like proteins (UBLs) have also been identified, including NEDD8, SUMO and ISG15, which share a characteristic three-dimensional fold with ubiquitin but are otherwise distinct. The UPS and UBL conjugation pathways have multiple essential biological roles and it is not surprising that their function, and often malfunction, are important factors in various human diseases[1], including numerous cancer types[2–4], cardiovascular disease[5], viral diseases[6] and neurodegenerative disorders[3]. These diseases may feasibly be targeted by modulating components of the UPS and UBL conjugation pathways using small-molecule inhibitors.

Indeed, the therapeutic potential of intervention in the UPS in cancer has been demonstrated by the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals), which was approved by the US Food and Drug Administration in 2003. Recent and ongoing research to elucidate the roles of other components of the UPS and UBL conjugation pathways has identified several enzymes that could be additional targets for therapeutic intervention using small-molecule inhibitors. In this Review, we first provide an overview of the enzyme classes in the UPS and UBL pathways that represent potential therapeutic targets, highlighting considerations that are important for drug discovery and recent progress in the development of small-molecule inhibitors. We then review recent developments in our understanding of the role of the components of the UPS and the UBL pathways in disease and their potential for therapeutic intervention.

Targets in the UPS and UBL enzymatic cascades

Ubiquitin and UBLs typically modulate protein function following covalent attachment to a primary amino group within a substrate protein, usually by forming an isopeptide bond with a lysine side-chain. The ubiquitin pathway is representative of this process and is shown in FIG. 1. The enzymatic cascade that results in protein ubiquitylation and degradation involves several distinct steps, each requiring a different class of enzyme.

In the first step, an E1 activating enzyme (primarily ubiquitin-activating enzyme (UAE; also known as UBA1), although ubiquitin-like modifier activating enzyme 6 (UBA6) can also activate ubiquitin[7,8]) consumes ATP and forms a high-energy thioester bond with the carboxyl terminus of ubiquitin. Ubiquitin is then passed to one of several E2 conjugating enzymes through a transthiolation

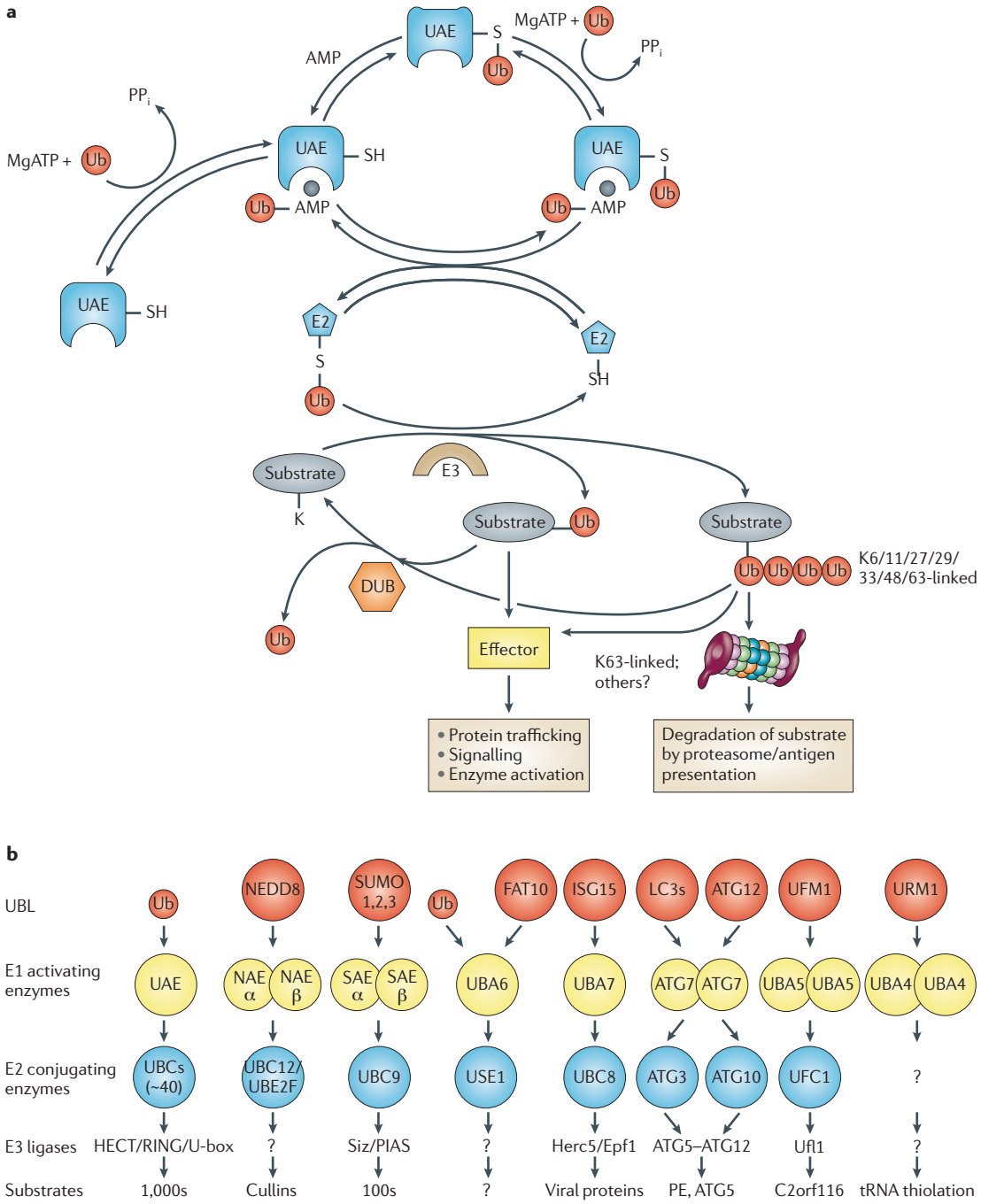

Figure 1 | **Overview of the enzymatic cascade involved in ubiquitin-like protein (UBL) conjugation and the ubiqitin–proteasome system (UPS). a** | Ubiquitin-activating enzyme (UAE) binds ATP and ubiquitin (Ub) to form a ternary complex consisting of E1–ubiquitin thioester with ubiquitin–AMP bound (see text for details). The thioester-bound ubiquitin is then passed to one of several E2 conjugating enzymes through a transthiolation reaction. The ubiquitin-charged E2 then forms a complex with an E3 ligase and a protein substrate to transfer ubiquitin to a lysine residue on the substrate. To mark substrates for degradation, multiple ubiquitins are similarly recruited to produce a K48-linked polyubiquitin chain. Following release from the E3, the proteasome recognizes the polyubiquitin chain, and the substrate is deubiquitylated and destroyed. Substrates marked with ubiquitin chains linked through lysines 6, 11, 27, 29 and 33 also seem to be primarily destined for degradation. Alternatively, substrates marked with monoubiquitin, linear ubiquitin chains or K63 ubiquitin chains are involved in signalling functions that are independent of the proteasome. A second E1 (ubiquitin-like modifier activating enzyme 6 (UBA6)) also activates ubiquitin, but the function(s) of this pathway are unknown. **b** | Nine classes of UBL and eight E1 activating enzymes participate in diverse biological pathways in humans. E1s, E2s and UBLs are structurally and mechanistically related but are unique to each pathway. Ubiquitin and some E2s are exceptions in that they can be used by both the UAE and UBA6 pathways. Ufl1 and C20orf166 were recently identified and reported by Tatsumi *et al*[139]. DUB, deubiquitylating enzyme; NAE, NEDD8-activating enzyme; PP_i, inorganic pyrophosphate; SAE, SUMO-activating enzyme.

Table 1 | **E1 activating enzymes, their cognate UBLs and biological roles***

E1	Structure	UBLs	Roles
UAE (UBA1)	Monomeric	Ubiquitin	Multiple, including protein homeostasis, transcriptional regulation and cell cycle progression
NAE (APPB1–UBA3)	Heterodimeric	NEDD8	Activation of cullin-based E3s
SAE (SAE1–UBA2)	Heterodimeric	SUMO1/2/3	Multiple, including protein stability, transcriptional regulation and cell cycle progression
UBA7	Monomeric	ISG15	Antiviral functions; possibly cell growth and differentiation
UBA6	Monomeric	FAT10	Antiviral functions
		Ubiquitin	Unknown
UBA4	Homodimeric	URM1	Antioxidant pathways; tRNA uracyl thiolation
UBA5	Homodimeric	UFM1	Unknown
ATG7	Homodimeric	ATG12	ATG5–ATG12 conjugate forms complex with ATG16 that functions as an E3 ligase for autophagic vesicle formation
		LC3	Autophagic vesicle formation

*For further information see REFS 15,142,143. NAE, NEDD8-activating enzyme; SAE, SUMO-activating enzyme; UBA, ubiquitin-like modifier activating enzyme; UBL, ubiquitin-like protein.

reaction. The ubiquitin-charged E2 then binds an E3 ligase and its protein substrate to transfer ubiquitin to an acceptor lysine residue on the substrate.

To mark substrates for degradation, multiple ubiquitins are similarly attached to produce a K48-linked polyubiquitin chain. On release of the substrate from the E3 ligase, the proteasome recognizes the polyubiquitin chain and the substrate is destroyed. Alternatively, substrates marked by monoubiquitylation or by polyubiquitin chains using different ubiquitin lysine sites are involved in functions such as protein trafficking, signalling and enzyme activation[2,9–11]. Deubiquitylating enzymes (DUBs) regulate the function of these various ubiquitin modifications and may, for example, rescue a substrate from degradation by removing a degradative ubiquitin signal or by changing or removing a non-degradative ubiquitin signal[12].

Although all UBL conjugation pathways are catalysed by structurally and functionally related enzyme cascades, UBLs participate in biologically distinct pathways (TABLE 1). Ubiquitylation is by far the most thoroughly studied UBL pathway and is 'information rich' in that numerous downstream receptors recognize and process differentially ubiquitylated proteins. Substrates that are polyubiquitylated with K48-linked ubiquitin chains are delivered to the 26S proteasome to be degraded into small peptides, with the ubiquitins released to be used again. Proteins that are polyubiquitylated with K63-linked ubiquitin chains are generally not degraded but are essential components of signalling pathways, for example, nuclear factor-κB (NF-κB)-dependent expression of inflammatory and immune genes. The function of ubiquitin chains that are linked through lysines 6, 11, 27, 29 and 33 (REF. 12) and of chains containing 'mixed' linkages is still emerging, although there is increasing evidence that non-K63 chains target proteins for degradation by the 26S proteasome[12].

With the exception of the proteasome (BOX 1; TABLE 2), the enzymes in the UPS and UBL conjugation pathways represent unprecedented drug targets and target classes. Indeed, significant advances in identifying small-molecule inhibitors of the UBL pathways have recently been achieved by targeting E1s, E2s, E3s, DUBs and the proteasome itself. Each UBL pathway enzyme class is mechanistically distinct. Also, E1s are highly specific for their respective UBLs and match individual UBLs with respective E2s. E3s are mostly associated with the ubiquitin pathway and are primarily responsible for substrate specificity and in some cases directly facilitate UBL transfer[13]. Similarly, DUBs also demonstrate a high degree of substrate specificity[14]. Therefore, there are a number of discrete E1–E2–E3 cascades that could provide opportunities to specifically target the aberrant signalling that is associated with particular diseases, and these are outlined below.

E1 activating enzymes

There are eight structurally and functionally related E1 activating enzymes that act at the apex of each of the nine classes of UBL conjugation pathways[15] (FIG. 1; TABLE 1). E1s can be classified on the basis of domain structure. The so-called canonical E1s include UAE, NEDD8-activating enzyme (NAE), SUMO-activating enzyme, UBA6 and UBA7, and the non-canonical E1s include ATG7, UBA4 and UBA5 (see REF. 15 for a review). All E1s share similar overall structural features and an ATP-dependent, multi-step mechanism for ubiquitin/UBL activation. Because E1s perform the first step of UBL activation, selective inhibition of an individual E1 would be expected to block the activation of each of the substrate UBLs for that E1, and subsequently block the function of affected UBL pathways.

E1s catalyse UBL activation by the multi-step process detailed in FIG. 2. In the first step, ATP and UBL bind together to form a UBL–acyl adenylate intermediate, releasing inorganic pyrophosphate[16]. UBL–AMP then reacts with the E1 active-site thiol to form an E1~UBL thioester (~ denotes a high-energy bond). A second ATP and UBL then bind the enzyme as in the first step to form a ternary complex that contains two UBL

Proteasome
The 26S proteasome is a protease complex that degrades polyubiquitylated proteins. It is composed of two subcomplexes: a barrel-shaped 20S core particle containing the protease active sites and two 19S regulatory particles that cap the barrel and control access of substrates to the core.

Nuclear factor-κB
(NF-κB). A transcription factor with a key role in regulating the immune response. NF-κB is involved in cellular responses to stimuli, including stress, cytokines, free radicals, ultraviolet irradiation and bacterial or viral antigens. Misregulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development.

Box 1 | Targeting the proteasome

In principle, the inhibition of ubiquitin-like protein pathway enzymes would provide specificity for the individual pathways and substrate proteins targeted. By contrast, inhibition of the proteasome blocks the final common step of the degradative pathways and is therefore a relatively nonspecific target. Nevertheless, the first clinically validated drug to target the ubiquitin–proteasome system (UPS) was the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals), which was approved in 2003. Owing to its success, several second-generation proteasome inhibitors are currently in development.

Each of the three types of proteasome active site — the caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) sites — use an amino terminal threonine as the catalytic amino-acid residue. The interactions of these active sites with various types of inhibitor are now known in atomic detail[98]. Bortezomib, as well as the newer inhibitors that are under clinical evaluation, use different chemical 'warheads'[73] to covalently modify the N-terminal threonine residue in one or more of the three sites. The properties of these agents, including enzyme inhibition kinetics and pharmacological differences, are summarized in TABLE 2.

For the most part, these compounds inhibit the β5 sites of both the constitutive proteasome and the immunoproteasome isoforms. Differences in enzyme kinetics may differentiate these proteasome inhibitors with regards to activity and safety; the irreversible nature of carfilzomib[99], ONX0912 (REFS 100,101) and NPI-0052 (REF. 102), compared with the slowly reversible binding of bortezomib[103] and CEP18870 (REF. 104), and the more rapidly reversible binding of MLN9708 (REF. 105), might result in differences in tissue distribution[106], and consequently in levels of UPS inhibition within different tumour types. Similarly, differences in specificity for the three enzymatic sites of the proteasome between these compounds[99,100,102–104,106] might also result in differences in activity in the different tumour types and in the safety of these proteasome inhibitors[105]. Furthermore, a number of selective inhibitors of the chymotrypsin-like subunit of the immunoproteasome have been reported, including PR957 (REF. 107) and IPSI-001 (REF. 108). Because of their selectivity, these compounds or similar inhibitors might have potential as therapeutic agents for autoimmune disorders.

molecules bound to the E1. This form of E1 is competent for transthiolation of the thioester-bound UBL to a pathway-specific E2 and is required for the downstream function of UBL conjugation.

As the enzymatic mechanism of E1s suggests, there are multiple points at which a small molecule could potentially inhibit UBL activation (BOX 2). For example, the inhibition of UBL–acyl adenylate formation could be achieved by preventing ATP from binding to E1 using an ATP-competitive small-molecule inhibitor similar to those now commonly used to inhibit kinases and other ATP-dependent enzymes. Alternatively, blocking E1~UBL thioester formation could be achieved by targeting the E1 active-site thiol. Blocking transfer of an E1 thioester-bound UBL to an E2 using molecules that compete with E1–E2 binding is yet another potential mechanism of inhibition[17]. Several strategies for inhibiting E1 function are now available and are beginning to demonstrate the feasibility of selective E1 targeting as a means to further understand UBL pathway biology (BOX 2).

E2 conjugating enzymes

E2s are responsible for transferring UBLs to substrate proteins and often function with a single or limited number of E3 ligases, although in some cases no E3 is required. In total, there are approximately 40 E2s, most of which associate with UAE and are involved in ubiquitin conjugation. The other E1s typically associate with one or sometimes two E2s[15] (FIG. 1; TABLE 1). Ubiquitin pathway E2s mediate ubiquitin chain assembly, determine ubiquitin-chain linkage topology and switch between chain initiation and elongation[18,19]. Together these factors determine the fate of ubiquitylated substrate proteins depending on whether they are mono- or polyubiquitylated and on the site(s) to which ubiquitin is conjugated.

Although several studies have linked E2s from different UBL pathways to cancer and other diseases, a fundamental question remains as to how the limited number of E2s pair with the much greater number of putative E3s. Unfortunately, despite structural and biochemical analyses of E2–E3 interactions, it is not yet possible to predict which E3 functions with a specific E2 and, as potent and selective E2 inhibitors are yet to be reported, our knowledge about their functions in cells is limited[20–22]. Despite these challenges, there are several emerging strategies that may yield inhibitors of E2s[20–22] and these are described in BOX 3, FIG. 4.

E3 ligases

Substrate selectivity of the UPS relies primarily on the specificity of the hundreds of E3 ubiquitin–protein ligases in the human genome. E3s are represented by four main classes: HECT domain E3s, U-box E3s[13], monomeric RING finger E3s[23] and multisubunit E3 complexes that contain a RING finger protein (FIG. 3). These classes differ in their protein interaction domains for binding to E2s, as well as in other domains related to substrate binding. The RING finger and U-box domains have an adaptor function in bringing ubiquitin-loaded E2 and the substrate together to promote ubiquitylation, whereas HECT E3s form a thioester intermediate with ubiquitin before transfer to a substrate. Individual E3s within these classes are responsible for the recruitment of specific substrate proteins to be tagged with ubiquitin or, in some cases, another UBL. Selective E3 inhibition may therefore enable direct targeting of an aberrant signalling pathway in cancer or other diseases.

In spite of the tremendous experimental and bioinformatics efforts that have taken place in the past 15 years, the annotation of E3 ligases that are associated with human disease is still in its infancy. The RING finger ligases alone constitute one of the largest enzyme groups in the cell (exceeding the kinases). The molecular mechanisms of E3 function have been elucidated primarily from structural studies. Identifying the substrate-binding surface or related sites mediating ubiquitylation activity, including sites of assembly for components of the E3 complexes, may facilitate the discovery of small-molecule inhibitors of E3s. Indeed, recent reports of inhibitors of cullin-RING ligases (CRLs; of which SCF is an archetype, see REF. 23 for review) represent significant advances. In one example, a chemical genetics screen in yeast for enhancers of rapamycin identified an inhibitor of SCF^{Met30} that directly bound to the F-box adaptor Met30 and diminished its binding to the core cullin–RING complex *in vivo*. Therefore, inhibition was achieved through the apparent failure to assemble a functional SCF complex[24].

HECT domain
A domain of ~350 amino acids found at the C terminus of HECT E3s. The HECT domain contains a catalytic cysteine residue that accepts ubiquitin from an E2 to form a thioester intermediate before transferring ubiquitin to a substrate lysine.

U-box
A domain comprising ~70 amino acids that possesses a tertiary structure resembling the RING finger domain. The major difference is that the U-box lacks the characteristic zinc-chelating cysteine and histidine residues of the RING finger. Consequently, U-box E3s use intramolecular interactions other than zinc chelation to maintain the RING finger motif.

RING finger
A domain present in most E3s that is defined by the consensus sequence C-X_2-C-$X_{[9-39]}$-C-$X_{[1-3]}$-H-$X_{[2-3]}$-C-X_2-C-$X_{[4-48]}$-C-X_2-C (where X means any amino acid). The RING domain coordinates two zinc ions.

Table 2 | **Properties of selected inhibitors of the proteasome and immunoproteasome**

Inhibitor	Development stage	Warhead	Target	Binding kinetics
Bortezomib	Approved for multiple myeloma, relapsed mantle cell lymphoma	Peptide boronic acid analogue[165,166]	• 20S proteasome: mainly β5 (IC_{50} values: β5 = 2.4–7.9 nM, β2 = 590–4,200 nM, β1 = 24–74 nM)[99,102,103] • Immunoproteasome: $β5_i$, $β1_i$ (REFS 73,108,167)	Slowly reversible (Half-life: 110 min)[103]
Carfilzomib	Phase III (relapsed multiple myeloma)	Peptide epoxyketone[99]	• 20S proteasome: mainly β5 (IC_{50} values: β5 = 6 nM, β2 = 3,600 nM, β1 = 2,400 nM)[99] • Immunoproteasome: $β5_i$ (REFS 73,168)	Irreversible
MLN9708	Phase I	Peptide boronic acid[103]	• 20S proteasome: mainly β5 (IC_{50} values: β5 = 3.4 nM, β2 = 3,500 nM, β1 = 31 nM)[103] • Immunoproteasome: not reported	Rapidly reversible (Half-life: 18 min)[103]
CEP18770	Phase I	P2 threonine boronic acid[104,106]	• 20S proteasome: mainly β5 (IC_{50} values: β5 = 3.8 nM, β2 = >100 nM, β1 = <100 nM)[104,106] • Immunoproteasome: not reported	Slowly reversible
NPI-0052	Phase I	Non-peptide bicyclic γ-lactam β-lactone[169]	• 20S proteasome: mainly β5, β2 (IC_{50} values: β5 = 3.5 nM, β2 = 28 nM, β1 = 430 nM)[102] • Immunoproteasome: not reported	Irreversible
ONX0912 (formerly PR047)[101]	Preclinical	Peptide epoxyketone[100]	• 20S proteasome: β5 (IC_{50} value = 36 nM) • Immunoproteasome: $β5_i$ (IC_{50} value = 82 nM)	Irreversible
PR957	Preclinical	Peptide epoxyketone[107]	• 20S proteasome: not reported • Immunoproteasome: $β5_i$-specific (20–40-fold more selective than β5 and $β1_i$)[107]	Irreversible
IPSI-001	Preclinical	Peptidyl aldehyde[108]	• 20S proteasome: β5 (K_i value = 105 μM), β1 (K_i value = 239 μM) • Immunoproteasome: $β5_i$ (K_i value = 1.03 μM), $β1_i$ (K_i value = 1.45 μM)[108]	Not reported

Cullin-RING ligases (CRLs). CRLs are a large family of multi-component E3s consisting of a core cullin protein bound to a RING finger protein (Rbx1/2), and an interchangeable substrate-binding adaptor protein. There are seven cullins and ~600 adaptors in the human genome. Modification of the cullin subunit by NEDD8 is required for activation of CRL E3 ligase activity.

SCF
SCF complexes are cullin RING ligases (CRLs) that catalyse the ubiquitylation of proteins targeted to the proteasome for degradation. SCF core subunits include the structural protein cullin 1, the RING-finger protein RBX1/2 and the adaptor protein Skp1. This core complex binds to one of the approximately 100 F-box proteins that are responsible for recruiting substrates. F-box proteins are named for the conserved 50 amino acid F-box domain that binds to SKP1. All CRLs, including SCFs, require NEDD8 modification of the cullin subunit for ligase activity.

In a second example, an inhibitor of SCF^{Cdc4} was identified in a biochemical screen that disrupted binding of the F-box protein CDC4 and its substrate, phosphorylated SIC1. Structural analysis showed that the compound bound CDC4 at a site 25 Å from the SIC1 binding site, resulting in a distortion of the substrate binding pocket and therefore achieving inhibition through an allosteric mechanism[25].

A third recent example demonstrates how thalidomide, a compound that was discovered over 50 years ago and was originally prescribed as a sedative until its use was discontinued, exerts its now notorious teratogenic effects. A search for primary targets of thalidomide identified cereblon (CRBN) as a thalidomide-binding protein[26]. CRBN forms a CRL complex with cullin 4 and the adaptor DNA damage-binding protein 1 (DDB1) that has E3 activity and is inhibited by thalidomide. Interestingly, a mutant of CRBN that does not bind thalidomide but retains E3 activity confers resistance to the teratogenic effects of thalidomide in animal models of embryonic development. This suggests that thalidomide exerts its teratogenic effects through CRBN. Together, these results not only shed light on the mechanism of thalidomide teratogenicity, but are also an example of the modulation of an E3 in a clinical setting using a small molecule (although with unintended and tragic consequences). Thalidomide is currently used clinically under strict control to treat multiple myeloma and leprosy, although the molecular target(s) in either of these contexts are unknown.

Collectively, these results indicate the feasibility of obtaining selective inhibitors of at least one important class of E3. However, difficulties remain given the overall protein subunit arrangements combined with the need for small-molecule inhibitors (generally a molecular mass of less than 1,000 Da) to bind and disrupt protein interfaces or otherwise allosterically affect ligase activity. The HECT ligases may ultimately prove to be more amenable to small-molecule intervention as these enzymes form covalent thioester intermediates with ubiquitin before transfer to lysine residues of target proteins. TABLE 3 provides several examples of E3s with disease implications along with inhibitors or other interventions that have been studied.

DUBs

DUBs are proteases that perform three major functions in UBL conjugation (see REF. 12 for a review). First, as UBLs are often translated as pro-proteins or as linear fusion proteins, DUB activity is required to cleave the C termini of UBLs and generate their mature forms. Second, DUBs can remove UBLs from modified substrates, attenuating UBL signalling functions and recycling free UBLs. Third, in the case of ubiquitin, DUBs have a polyubiquitin chain editing or 'proof reading' function. In mammals, there are nearly 100 DUBs belonging to five different families, the majority of which are cysteine proteases; the remainder consists of a small number of zinc metalloproteases. Historically, it has been problematic identifying drug-like inhibitors of cysteine proteases, whereas the general class of metalloproteases has been more amenable to inhibition using small-molecule drugs (BOX 4). Recently, a global proteomic analysis identified 774 candidate interacting proteins associated with 75 DUBs, allowing placement of

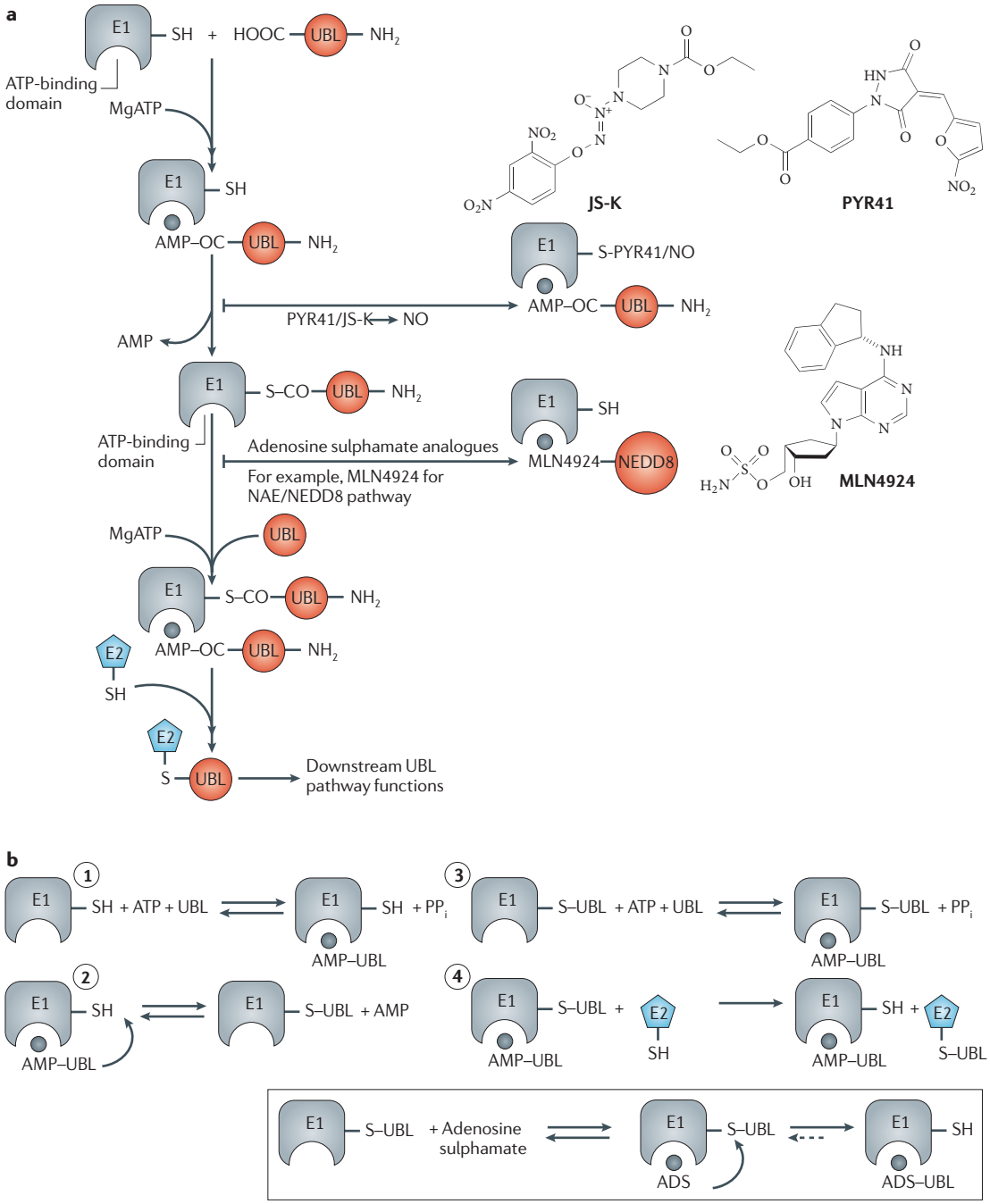

Figure 2 | **Mechanisms of E1 inhibitors identified in studies of different ubiquitin-like protein (UBL) pathways.** **a** | In the first step of UBL activation, E1s bind ATP and a cognate UBL and catalyse the formation of a UBL carboxy-terminal acyl adenylate. The E1 catalytic cysteine then attacks the UBL–adenylate to form a thioester with the C terminus of the UBL. The E1 subsequently binds a second ATP and UBL, again forming a UBL–adenylate and resulting in the formation of a ternary complex consisting of an E1–UBL thioester with UBL–adenylate bound to it. This form of the E1 is fully competent to transfer thioester-bound UBL to a cognate E2 enzyme and initiate the downstream effects of UBL signalling. Small molecules, including the pyrazone derivative PYR41, JS-K and MLN4924, use distinct mechanisms to block this process at different stages of the E1 reaction cycle (see BOX 2 for further details). **b** | Substrate-assisted mechanism-based E1 inhibition. E1s use a multistep mechanism to form a ternary complex consisting of an E1~UBL thioester (~ denotes a high-energy bond) with UBL–adenylate bound to it (steps 1–3). This form of E1 is competent for UBL transfer to an E2 by a transthiolation reaction (step 4). The NEDD8-activating enzyme (NAE)-selective inhibitor MLN4924 and related adenosine sulphamate analogues are mechanism-based inhibitors of E1s and form covalent UBL–inhibitor adducts *in situ*, catalysed by the E1 itself. Inhibitors of this class bind exclusively to the UBL thioester form of E1 shown in step 2 and attack the thioester bond to yield the UBL–inhibitor adduct. The UBL–inhibitor adduct mimics UBL–adenylate, the first intermediate in the E1 reaction cycle, but cannot be further used in subsequent intra-enzyme reactions. The stability of the UBL–inhibitor adduct within the E1 active site adenylation domain blocks enzyme activity.

Rapamycin
Rapamycin (sirolimus) is a macrocyclic antibiotic produced by a bacterium isolated from soil on Easter Island. Rapamycin binds the cytosolic protein FK-binding protein 12 (FKBP12). The rapamcyin–FKBP12 complex inhibits the mTOR (mammalian target of rapamycin) pathway by directly binding mTOR complex1 (mTORC1).

Cysteine proteases
This class of protease uses a cysteine thiol group in its catalytic mechanism. Deprotonation of the cysteine sulphydryl by an adjacent residue (usually histidine) is followed by nucleophilic attack on the peptide carbonyl carbon. A thioester linking the new C terminus to the cysteine thiol is an intermediate of the reaction.

Zinc metalloproteases
A class of protease for which the active sites include two histidine residues that coordinate a zinc ion. During catalysis, the Zn^{2+} promotes attack of the peptide carbonyl carbon by the oxygen atom of a water molecule at the active site. An active site base facilitates this reaction by extracting a proton from the attacking water molecule.

Box 2 | **Targeting E1 activating enzymes**

Inhibition of UAE through covalent inactivation

The active-site thiol that is present in E1s is essential for ubiquitin-like protein (UBL) activation and offers a potential opportunity for inhibition using small molecules (FIG. 2a). PYR41 is a pyrazone derivative identified in an *in vitro* high-throughput screen for inhibitors of HDM2-dependent p53 ubiquitylation. Characterization of the inhibitor revealed that the nitrogen dioxide group on the furan ring of PYR41 covalently modified the ubiquitin-activating enzyme (UAE) active site cysteine[109]. Importantly, the compound did not demonstrate inhibitory activity against other thiol-dependent enzymes, including several E2s[109]. The effects in cells treated with PYR41 included inhibition of cytokine-induced nuclear factor-κB (NF-κB) activation, and stabilization of p53 coupled with the induction of p53-dependent transcription[109]. The related dioxopyrazolidine, PYZD4409, showed similar UAE inhibitory properties *in vitro* and also demonstrated antileukaemic activity in a mouse cancer model[110]. The potential therapeutic value of targeting UAE is further underscored by the unexpected finding that the nitric oxide (NO)-producing prodrug JS-K inhibited UAE~ubiquitin thioester formation (~ denotes a high-energy bond) through an interaction between NO and the UAE active-site thiol[111]. Consistent with other UAE inhibitors, the downstream effects of JS-K treatment included decreased levels of total ubiquitylated proteins and increased p53 expression.

Inhibition of NAE through adduct formation

Acyl-adenylate analogues of UBL–AMP are known to be potent and selective E1 inhibitors but are not suitably drug-like for delivery to intracellular targets. A new approach using small molecules to form inhibitory UBL–AMP mimetics *in situ* overcomes this limitation. Inhibition of the NEDD8 pathway was recently demonstrated using the small-molecule NEDD8-activating enzyme (NAE) inhibitor, MLN4924 (REFS 112,113). MLN4924 is an adenosine sulphamate analogue that binds to the nucleotide-binding pocket of the NEDD8 thioester form of NAE and forms a covalent adduct with NEDD8 by a mechanism referred to as 'substrate-assisted inhibition' (FIG. 2b). Because NEDD8 conjugation of cullin proteins is required for the ubiquitin ligase activity of the cullin-RING finger ligases (CRLs)[23,112,114,115], (FIG. 3), blocking NAE results in the inhibition of CRL activity and the stabilization of CRL substrates, some of which are important for cancer cell growth and survival (TABLE 3). For example, NAE inhibition results in the stabilization of CDT1, a substrate of the $CRL1^{SKP2}$ and $CRL4–DDB1^{CDT2}$ ligases, leading to DNA re-replication and apoptosis in proliferating cells[112]. Studies using NF-κB-dependent human cancer models have demonstrated increased levels of the $CRL1\beta^{TRCP}$ substrate plκBα and inhibition of NF-κB activity and apoptosis[116,117], suggesting the feasibility of NAE inhibition for the treatment of disease that is associated with constitutively active NF-κB signalling[112]. Notably, analogues of MLN4924 form similar adducts with other UBLs and catalyse their cognate E1s, including UAE and SUMO-activating enzyme, suggesting that substrate-assisted inhibition may prove useful for targeting other UBL pathways[118].

previously unstudied DUBs within cellular protein complexes as well as putative biological pathways[27]. However, analogous to the challenges mentioned above for E3s, it is unclear at present whether defining the DUB interactome will reveal additional drug targets. Nonetheless, the biological roles of DUBs make them attractive targets for pharmaceutical intervention.

The clinical utility of proteasome inhibitors and our increasing understanding of the enzyme mechanisms of UBL conjugation and deconjugation hint at the increasing likelihood of identifying drug-like, small-molecule inhibitors of these novel targets. Ongoing research to elucidate the roles of specific enzyme components of the UPS and UBL conjugation pathways has identified several potential targets in specific disease settings. In the next sections, we detail several examples in which therapeutic opportunities may exist.

UPS and UBLs: therapeutic opportunities

The degree to which diverse biological processes are regulated by the UPS and UBL conjugation pathways is extraordinary. It is therefore not surprising that misregulation of these pathways is implicated in a growing number of diseases. Oncology is an area for which targeting this system is particularly exciting and for which proteasome inhibition is a valid approach. Here, we focus on additional examples in which UPS and UBL pathway enzyme targets are tightly linked with specific pathologies.

NF-κB, inflammation and immunity. The complexity of protein ubiquitylation in signal transduction is illustrated by the fundamental role of ubiquitin in the activation of the transcription factor NF-κB, which has a key role in inflammation and immunity[28,29]. There are differences in the activation of NF-κB by different ligands, that is, binding to receptors for tumour necrosis factor (TNF), interleukin-1 (IL-1) and T cell receptor, which include the use of linear and K63-linked ubiquitin chains.

The study of the multiple roles of ubiquitin in the enzymatic cascade from receptor–ligand binding to the activation of the expression of NF-κB-dependent genes is complicated by several issues (FIG. 5). First, multiple enzymes catalyse ubiquitylation at the same target lysine in a protein. For example, the A20 (also known as TNFα-induced protein 3 or TNFAIP3) and ITCH ligases trigger ubiquitylation and degradation of the RIP kinase, a vital component of the ligand-dependent activation of NF-κB. Second, the lability of ubiquitylated species is a problem due, for example, to regulatory deubiquitylation. Third, there are different types of polyubiquitin chain formation at single ubiquitylation sites (lysine residues), such as K63-linked or linear chains, and some proteins also have multiple ubiquitylation sites. Fourth, there are experimental difficulties in studying ubiquitylation reactions, such as the low abundance of ubiquitylated proteins and the need to experimentally use mutated ubiquitins with non-physiological conformational changes[29].

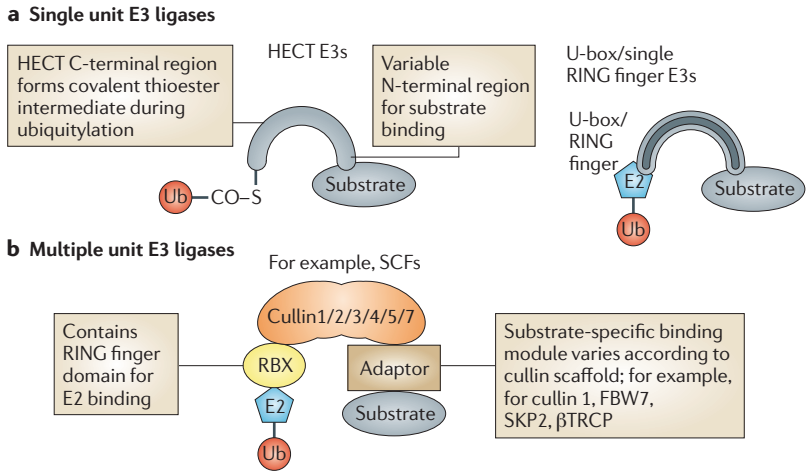

Figure 3 | **Structural differences among various types of E3 ligases.** There are four major classes of ubiquitin ligase: HECT domain proteins, U-box proteins, monomeric RING finger E3s, and multisubunit E3 complexes that contain a RING finger protein. **a** | HECT domain E3s use a unique mechanism in which ubiquitin (Ub) is transferred from an E2 to a conserved cysteine residue within the E3 via transthiolation and is then transferred from the E3 to a substrate amino group. All other types of E3 facilitate transfer of Ub from a charged E2 directly to a substrate. **b** | The RING finger motif comprises a Zn^{2+} binding domain that is required for E2 binding. The U-box also binds E2s and is structurally similar to the RING motif, but does not bind metal ions. Monomeric RING finger E3s and U-box E3s bind to both the substrate and the E2. In multimeric RING finger complexes, the RING finger protein binds the E2 while other proteins in the complex bind the substrate. These multimeric complexes include the anaphase promoting complex/cyclosome and cullin-RING ligases (CRLs). A unique aspect of CRLs is the requirement for modification of the cullin subunit by NEDD8 for ubiquitin ligase activity (BOX 2). RBX, RING-box protein.

The recent discovery of the importance of linear ubiquitin chains in NF-κB activation extends the complexity of the regulation of the system. A linear ubiquitin chain-assembly complex (LUBAC) conjugates head-to-tail-linked linear polyubiquitin chains to target proteins. LUBAC is involved in the physiological regulation of the canonical NF-κB pathway by binding to NEMO (NF-κB essential modulator; also known as IKKγ) and in the conjugation of linear polyubiquitin chains on to specific lysine residues of NEMO[30]. The recruitment of LUBAC to the TNF receptor 1 (TNF-R1) signalling complex (TNF-RSC) is stimulation-dependent and requires TRADD, TRAF2 and cellular inhibitors of apoptosis (IAPs), but not NEMO or RIP1. It seems that LUBAC is recruited to the TNF-R1 by cellular IAP-generated ubiquitin chains and generates linear ubiquitin chains to increase the efficiency of NF-κB and Jun N-terminal kinase activation. Activation of NF-κB increases TNF-dependent gene expression, whereas activation of Jun N-terminal kinase inhibits TNF-mediated apoptosis. The attraction of NEMO to the TNF-RSC is increased by LUBAC, and the enzymatic activity of LUBAC seems to stabilize the TNF-RSC[31].

This biochemical mechanism is facilitated by the fact that the UBAN (ubiquitin binding in NEMO) motif of NEMO selectively binds linear ubiquitin chains. The specific amino-acid residues of NEMO that are involved in binding linear ubiquitin chains have been reported to be mutated in humans and result in X-linked ectodermal dysplasia and immunodeficiency[32]. The binding of polyubiquitin chains to NEMO is interesting in that the C-terminal zinc finger of NEMO can bind ubiquitin as well as UBAN. Although neither UBAN nor the zinc finger show any preference for K63-linked chains, together the domains form a high-affinity K63-specific ubiquitin-binding domain. This suggests that the main function of the C-terminal half of NEMO is to specifically bind K63-linked polyubiquitin chains. The binding of NEMO to linear polyubiquitin chains is dependent on the UBAN alone and does not require the presence of the zinc finger[33]. Furthermore, unanchored K63-linked polyubiquitin chains can directly activate TAK1 and IKK kinases. This indicates a new role for free ubiquitin chains in activating kinases. Cleavage of the unanchored K63-linked ubiquitin chains prevents TAK1 and IKK activation[34]. Further research may show that unanchored ubiquitin chains are generated as 'second messages', as with cAMP.

Whatever the mechanism(s), protein ubiquitylation/deubiquitylation is at the heart of the inflammatory and immune responses, together with protein phosphorylation/dephosphorylation. Therefore, ubiquitylation should be amenable to therapeutic intervention for inflammatory and immune disorders, but at what level? Although specific E3s such as cellular IAPs are obvious targets, the E2s that are involved in K63 ubiquitin chain synthesis (for example, UBC13/UEV1A or UBCH5) or in linear chain assembly (for example, UBCH5, E2-25 kDa and UBCH7), might be targeted with the provision that multiple signalling pathways may be compromised by inhibitors of these enzymes. It seems that E3s use different E2s to catalyse distinct types of ubiquitin tagging. For example, RING finger IAPs seem to bind to UBCH5A, UBCH5C, UBC7, UBC8 and UBC13/UEV1A[35]. It is also generally thought that, although E3s are the ultimate arbiters of substrate selection for ubiquitylation, the E2s determine what type of polymeric ubiquitin chain will be formed. It remains to be seen how E3s select appropriate E2s for customized polymeric ubiquitin chain assembly on specific target proteins.

In addition, the E3s are key regulators of immune functions. Several E3s, including c-CBL, CBL-b, GRAIL, ITCH and NEDD4, have been shown to negatively regulate T cell activation by contributing to T cell apoptosis. However, the HECT ligase AIP2 positively regulates T cell activation by enhancing T cell proliferation and IL-2 production to suppress apoptosis. AIP2 interacts with and promotes ubiquitin-mediated degradation of EGR2, a zinc finger transcription factor, to suppress EGR2-mediated FasL expression and activation-induced death of T cells[36]. Careful selection of the appropriate E3 target for inhibition might ameliorate or block T cell activities.

The taxing biological issue with disease-related consequences is what aspect of NF-κB signalling should be clinically interrupted. For example, normal NF-κB functions are essential for the immune response against microbial infections, but continuous NF-κB activation can lead to inflammation and cancer. For this reason,

Box 3 | **Targeting E2 conjugating enzymes**

Several studies have linked E2s from different ubiquitin-like protein (UBL) pathways to cancer and other diseases, including UBE2Q2 in head and neck squamous cell carcinoma[119]; the SUMO E2 UBC9 in ovarian carcinoma, melanoma, lung adenocarcinoma and breast cancer[120–122]; UBE2T in lung cancer[123]; and UBCH10 in chromosomal instability and tumour formation[124,125]. In all cases, an E2 must engage its respective E1 to be charged with a UBL through transthiolation. In the ubiquitin pathway, E2s usually require an E3 for substrate ubiquitylation and therefore must also bind to an E3–substrate complex. For conjugation to a protein substrate to occur, a UBL is transferred from the E2 thiol active site to the amino group of a substrate acceptor lysine residue, which must be positioned for nucleophilic attack on the carbonyl group in the E2–UBL thioester bond (FIG. 4).

Each of these E2 interactions offers potential opportunities for selective inhibition. For example, a synthetic peptide corresponding to the 26-residue amino terminus extension of UBC12 (called UBC12N26) has been shown to compete for binding of UBC12 to the NEDD8-activating enzyme (NAE), thereby blocking transfer of NEDD8 to downstream targets[17]. Alternatively, recent published findings indicate that E2–E3 recognition, although necessary, is not sufficient for ubiquitin transfer[126]. In one example, the G2BR domain of the RING finger E3 gp78 binds UBE2G2 with high affinity and allosterically activates ubiquitylation activity on formation of the E2–E3 complex[127]. Targeting allosteric activation of an E2 would prevent subsequent ubiquitylation of substrate proteins, thereby blocking a specific UBL pathway. Finally, a conserved asparagine residue (exemplified by asparagine 79 in UBC13) in E2s has been shown to be crucial for UBL transfer and is thought to form the oxyanion hole required to stabilize the E2 thioester–substrate transition state intermediate[128]. Targeting this site with a small-molecule effector may offer yet another approach for inhibition.

tight negative regulation of NF-κB activity occurs through mechanisms involving DUBs. The first DUB that was shown to be involved in the NF-κB system was CYLD, a tumour suppressor involved in a neoplasm called cylindromatosis. The enzyme has a great specificity for K63-linked ubiquitins[37] and the catalytic site involved in the cleavage of K63-linked chains is often mutated in this cancer. Additionally, CYLD (like isopeptidase T) preferentially cleaves unanchored K63-linked polyubiquitin chains and therefore supports the role of these chains in protein kinase activation in the NF-κB pathway[34]. Another DUB with a key role in the negative regulation of the NF-κB response is A20. This enzyme has dual functions in removing K63-linked chains from RIP1 and TRAF6, and uses its ubiquitin ligase activity to attach K48-linked chains to RIP1 for proteasomal degradation[38].

The inhibition of CYLD and/or A20 could enhance the inflammatory or immune response, but the prolonged consequences are unclear and await assessment in animal models and in early clinical trials. Alternatively, prevention of TNF-dependent NF-κB activation by linear chains through the inhibition of one or more of the E2s (UBCH5, E2-25kDa or UBCH7) may suppress NF-κB activity and inflammatory and immune responses — but again, with what long-term clinical price? For context, the use of steroids for the long-term suppression of various inflammatory conditions is routine, with generally acceptable side effects.

There might be multiple mechanisms to normally ensure the termination of NF-κB activation. However, it is clear that the intracellular ubiquitin-editing protein A20 has a key role in the negative feedback regulation of NF-κB signalling (through TNF and Toll-like receptors (TLRs)) in response to multiple stimuli. The importance of A20 in the negative regulation of NF-κB signalling through TLRs is illustrated by recent findings that A20 disrupts the ubiquitin ligase activities of TRAF6, TRAF2 and CIAP1 by preventing interactions with the E2s UBC13 and UBCH5c and with another regulatory protein, TAX1BP1, to trigger the A20-dependent ubiquitylation of the E2s and their proteasomal degradation[39]. Recent genetic studies demonstrate that several mutations at the human A20 locus relate to immunopathologies such as Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes. The gene encoding A20 seems to be a susceptibility gene for many different immune disorders[40]. Finally, and a caveat to any inhibition of A20, is the role of A20 as a tumour suppressor[41]. Acute transient inhibition of A20, by promoting NF-κB activation, may be useful in resolving infections, but chronic inhibition may promote cancer.

During the innate immune response to some microorganisms, the binding of lipopolysaccharide to the TLR receptor complex TLR4–MD2–CD14 leads to the recruitment of the adaptors Mal, MyD88, TRAF6 and several IRAK kinases. TRAF6 is activated by IRAK to cause the attachment of K63 chains to both TRAF6 and IRAK. These chains are recognized by TAB2 and NEMO, leading to TAK1-mediated phosphorylation and activation of the IKK kinase complex. The A20 DUB activity is directed at TRAF6 to downregulate NF-κB activities[40]. It is possible that inhibitors of the DUB activity of A20 may enhance antimicrobial activity in response to intracellular bacterial infections, such as *Salmonella*, *Listeria* and *Typhimurium*.

There are further consequences of manipulating the NF-κB system other than the regulation of inflammatory and immune responses. The system is intricately linked to cell death. For example, internalization of the TNFR1 complex can give rise to further complexes in the cytosol that are dependent on the TRADD component of the TNFR1 complex (to give complex IIA) or RIP1 (to give complex IIB). Complex IIA formation involves the recruitment of the protein Fas-associated death domain (FADD) and caspase 8 to trigger apoptosis, whereas complex IIB is formed when the FADD pathway is blocked by, for example, viral proteins or chemical inhibitors of apoptosis, and mediates cell death by

Table 3 | **Selected E3 ligases, substrates, associations with human disease and mechanistic targeting**

E3 ligase	Substrate	Disease association	Inhibitor/intervention investigated
MDM2 (HDM2)[13]	p53, p27 (REF. 144)	Multiple cancers, including breast and lung, oesophageal carcinomas, glioblastomas and malignant melanomas	Nutlins[145] and RITA[146] to promote p53 stabilization; parthenolide to promote MDM2 ubiquitylation[147]
$CRL^{SKP2/\beta TRCP/FBW7}$ (REFS 13,148)	p21, p27, cyclin D, β-catenin[149], IκBα[150]	Multiple cancers and other disorders linked to the NF-κB pathway	NAE inhibition results in loss of NEDD8 modification of cullin proteins and inactivation of CRL activity
IAP[151]	Various substrates involved in apoptosis and signalling	Various cancers, including oesophageal, liver and lung, ovarian carcinoma and MALT lymphoma	SMAC mimetics neutralize IAP inhibition of caspase-induced apoptosis and promote IAP auto-ubiquitylation and degradation
XIAP[152]	AIF, MEKK2, TGFβ-activated kinase[152,153]	TGFβ stimulation of NF-κB in breast cancer[153]; acute myeloid leukaemia[154,155]	Triptolide induction of apoptosis in XIAP-overexpressing AML cells[154]; antisense compound AEG35156 (REF. 156)
E6-AP[157]	p53	Cervical cancer through HPV16/18 infection[157]; mutations associated with Angelman's syndrome[158]	Expression of mRNA decay factor TTP stabilizes p53 through E6–AP targeting in HPV-transformed cervical cancer
VHL[159]	HIF1α[160], HIF2α[161]	VHL syndrome; inactivation associated with renal cell carcinoma[159]	Bioengineered VHL protein to increase HIF degradation[162]
Parkin[55,163]	Synphilin 1, Parkin[55]	Parkinson's disease	Nitric oxide inhibits E3 activity
BMI1/RING1A[164]	TOP2A	B and T cell leukaemias, various other cancer cell lines	PRT4165 inhibits BMI1/RING1A activity and enhances potency of TOP2 drugs

AIF, apoptosis-inducing factor; AML, acute myeloid leukaemia; CRL, cullin-RING ligases; HIF, hypoxia-inducible factor; HPV, human papillomavirus; IAP, inhibitor of apoptosis; MALT, mucosa-associated lymphoid tissue; NAE, NEDD8-activating enzyme; NF-κB, nuclear factor-κB; TGF, transforming growth factor; TOP2A, topoisomerase (DNA) II alpha; TTP, tristetraprolin; VHL, von Hippel–Lindau.

necroptosis. In this pathway, complex IIB contains both RIP1 and RIP3, which by mutual co-phosphorylation can activate enzymes involved in metabolism. Activated RIP3 increases the activities of enzymes involved in glycolysis and mitochondrial oxidative phosphorylation and, in some cases, can cause the production of reactive oxygen species to mediate necrotic cell death. It is assumed that complex IIB contains deubiquitylated RIP caused by CYLD and A20. Necroptotic cell death may therefore be compromised by inhibition of these DUBs, for example, in virally infected cells, although complex IIA-dependent apoptotic cell death may still operate[42,43]. Again, to show the complexity of these cellular responses to various stresses, the necrostatin inhibitors of necroptosis[44] can reduce tissue damage in brain ischaemia and myocardial infarction[45]. Therefore, it is possible that CYLD and A20 inhibitors may prevent tissue necrosis in appropriate clinical indications, such as following stroke and heart attack, for which it is currently difficult to limit tissue damage.

Bone disorders. The roles of NF-κB in inflammation and the immune response, and therefore the usefulness of ubiquitin pathway inhibitors, could be extended to osteolytic disorders. Osteoclasts are responsible for bone resorption and have a pivotal role in the pathogenesis of osteolytic disorders. NF-κB signalling pathways are strictly regulated to maintain bone homeostasis through cytokines, such as RANKL, TNF-α and IL-1, which differentially regulate classical and/or alternative NF-κB pathways in osteoclastic cells. Abnormal activation of NF-κB signalling in osteoclasts has been associated with excessive osteoclastic activity, and is frequently observed in osteolytic conditions, including periprosthetic osteolysis, arthritis, Paget's disease of bone and periodontitis. NF-κB modulators such as parthenolide and NEMO-binding domain peptide demonstrate therapeutic effects on inflammation-induced bone destruction in mouse models[46]. Therapeutic intervention in the activities of the UPS may slow disease progression of osteolytic disorders that cause debilitating disease in the ageing population, for example, Paget's disease of bone.

The UPS and heart disease. The role of the UPS in cardiac function and disease is again complex and sometimes conflicting. Proteasome inhibitors have been used in the experimental manipulation of heart disease. The outcomes so far indicate, as might be expected, that the effects of proteasome inhibitors are concentration dependent, with lower concentrations mediating cytoprotective effects and higher concentrations being toxic. For example, studies on non-toxic proteasome inhibition in neonatal rat cardiac myocytes show upregulation of antioxidative enzymes, which confers cardioprotection. This upregulation is a response to mild proteasome inhibition by antioxidative transcription factor NF-E2-related factor 2 (NRF2)-dependent transcriptional activation of an antioxidant response element (ARE) in the superoxide dismutase 1 (SOD1) promoter. The induction of antioxidative enzymes and cytoprotection was evident in cardiomyocytes from wild-type mice, but was completely abolished in cells from *Nrf2*-knockout animals[47].

Autophagy
Literally means 'self-eating'; a highly regulated catabolic process in which cellular proteins and organelles are sequestered in a characteristic double-membrane vesicle called an autophagosome and are then degraded following vesicular fusion with a lysosome.

Endosome–lysosome pathway
Endosomes are membrane-bound vesicles that are involved in protein transport between the plasma membrane, Golgi and lysosomes. In the endocytic pathway, internalized molecules are delivered to early endosomes, where efficient sorting occurs. Some molecules, including recycling receptors, are shunted back to the plasma membrane to be reused. Others, including downregulated receptors, are transported to late endosomes and lysosomes for degradation.

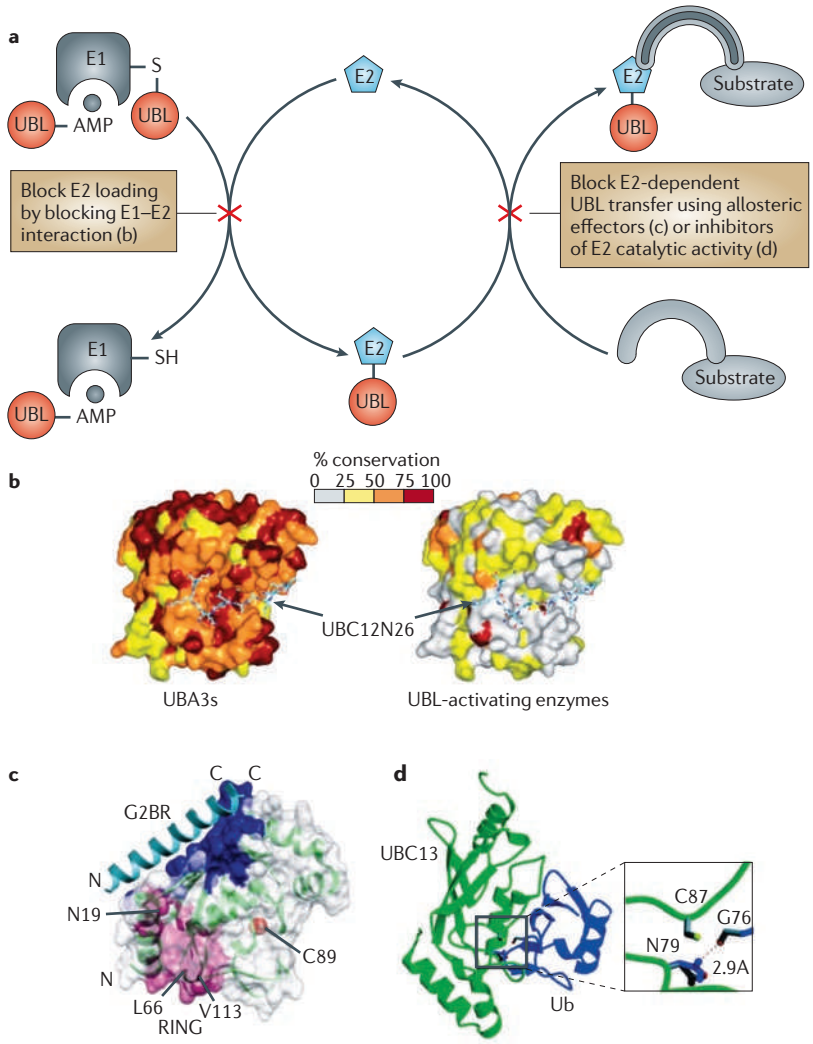

Figure 4 | **Molecular interactions of E2s suggest potential modes of inhibition.** **a** | E2s engage in a sequence of highly specific interactions to faithfully transfer a ubiquitin-like protein (UBL) to a substrate. E2s first bind their respective E1 to receive an activated UBL through a transthiolation reaction. In most cases, the E2–UBL thioester then binds a specific E3 or E3–substrate complex. During catalysis to protein substrates, the UBL is transferred from the E2 thiol active site to the amino group of a substrate acceptor lysine residue that is positioned for nucleophilic attack on the E2–UBL thioester bond. Each of these E2 interactions offers potential opportunities for selective inhibition as illustrated by the following examples. **b** | Targeting E2~UBL thioester formation by blocking E1–E2 protein–protein interaction. A synthetic peptide called UBC12N26 corresponds to the 26-residue amino terminus extension of UBC12 that specifically binds the NEDD8-activiating enzyme (NAE). UBC12N26 competes for binding of UBC12 to NAE, thereby blocking transthiolation of NEDD8 to UBC12 and inhibiting downstream NEDD8-dependent events. **c** | Targeting E3-dependent E2 allosteric activation. In some cases, E2–E3 binding is necessary but not sufficient for optimal UBL transfer to a substrate. For example, the E3 gp78 binds the E2, UBE2G2, through a RING finger and a second domain called G2BR that binds the backside of UBE2G2, which is opposite from the catalytic cysteine and distal from the RING binding surface. Blocking G2BR interaction with UBE2G2 would inhibit allosteric activation of the E2 and prevent subsequent ubiquitylation of substrate proteins. **d** | Targeting catalysis of UBL transfer. A conserved asparagine residue in E2s (exemplified by asparagine 79 in UBC13) has been shown to be crucial for UBL transfer and is thought to form the oxyanion hole required to stabilize the E2 thioester–substrate transition state intermediate. Targeting this site with a small-molecule effector may offer yet another approach for inhibition. Ub, ubiquitin.

More understanding of the UPS in the pathophysiology of heart disease is needed to contemplate new routes for therapy. It seems that pressure overload, ischaemic heart disease or genetic mutations in contractile proteins that cause heart failure are accompanied by elevation in levels of misfolded proteins, which may be removed by the UPS and through autophagy. However, proteasome inhibitors may be useful for the treatment of cardiac hypertrophy and ischaemic heart diseases[48]. A complication is that impaired proteasome function is commonly associated with myocardial ischaemic injury, but recent evidence also supports a cardioprotective role for proteasome inhibitors in myocardial ischaemia[49]. The loss of cardiomyocytes is a key problem in the development of cardiovascular disease. Two main processes mediate cardiomyocyte loss: necrosis and apoptosis. In contrast to necrosis, apoptosis is a relatively well-understood, regulated process that is essential in normal development and tissue homeostasis. Tight regulation of both processes is crucial, especially in post-mitotic cells lacking regenerative capacity, such as cardiomyocytes and neurons. The UPS is involved in the regulation of cardiomyocyte apoptosis[50] and possibly necrosis.

As in cerebrovascular accidents in the brain, two different processes might be controlled by the proteasome: upregulation of the expression of gene products that ameliorate oxidative damage in surviving cardiomyocytes by proteasome inhibition, and the activation of cardiomyocyte death pathways. The clinical outcome will be dependent on the balance between these proteasome (and general UPS) activities. Caution is needed clinically, as cardiotoxicity has been reported in patients treated with the proteasome inhibitor bortezomib[51].

In spite of these complexities, consideration should be given to the use of proteasome inhibitors and other modulators of the UPS for viral myocarditis. The primary intracellular protein degradation systems, both the UPS and autophagy, seem to regulate successive stages of viral infectivity. Viral myocarditis, such as that caused by coxsackievirus B3, progresses in three distinct stages: acute viral infection, immune cell infiltration and cardiac remodelling. The UPS has a central role in each of these stages of viral infection and might be modulated to slow viral disease progression and heart disease[52].

Chronic neurodegenerative disease. As expected, ubiquitin-dependent processes, including the degradation of ubiquitylated proteins by the 26S proteasome, by autophagy and in the endosome–lysosome pathway, have central roles in neuronal development, homeostasis and disease. These catabolic systems are essential for neuronal activities, including synaptogenesis, cell–cell interactions (for example, neuromuscular junction formation[53]) and synaptic plasticity[54]. In the adult central and peripheral nervous systems, which are based predominately on non-dividing cells, protein ubiquitylation and deubiquitylation are essential for neuronal survival. Most age-related chronic neurodegenerative diseases involve the accumulation of proteins in aggregates, frequently as paranuclear inclusions (FIG. 6). In every case, proteins within the inclusions are ubiquitylated.

Box 4 | **Targeting deubiquitylating enzymes**

Enzymes that reverse the action of the ubiquitin conjugation cascade, the deubiquitylating enzymes (DUBs), are attractive targets for drug discovery because of the various ubiquitin-mediated biological processes that they regulate[14,27,129,130]. In mammals, there are nearly 100 DUBs belonging to five different families. Four of these families — the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour domain proteins and Machado–Joseph disease protein — are all papain-like cysteine peptidases. Many potent inhibitors exist for enzymes of this general mechanism, but historically there has been limited success in turning these leads into drugs. Nevertheless, a desire to clear this hurdle persists because of the many biological roles that DUBs have. Individual cysteine peptidase DUBs have been associated with pathways of importance in specific diseases, such as USP1 with the Fanconi anaemia DNA repair pathway[131]; USP6 with aneurysmal bone cysts and with a wider role in mesenchymal tumours[14]; and USP7 with non-small cell lung adenocarcinoma[14,132]. Loss of the deubiquitylating activity of the DUB CYLD is associated with cylindromatosis[133], and UCH-L1 has been associated with Parkinson's disease[134]. Despite the difficulty so far in turning these leads into drugs, there are glimmers of hope. For example, a non-covalent inhibitor of the papain-like protease from the SARS coronavirus, which acts as a DUB for the virus, has been shown to block virus replication; importantly, this compound demonstrates specificity, inhibiting the pathogenic DUB but not host DUBs[2,135–137].

The fifth family of DUBs, the JAMM (Jab1/MPN domain-associated metalloisopeptidase) domain proteins, are zinc metalloisopeptidases[14]. Historically, the general class of metalloproteases has been more amenable to small-molecule drugs. Several members of the JAMM class, including POH1, CSN5, AMSH and BRCC36, might make good drug targets. POH1 functions as part of the 19S cap of the 26S proteasome to trim polyubiquitin chains from substrates that are destined for degradation. Targeting this enzyme could yield new chemical classes of proteasome inhibitor (BOX 1). CSN5 is a subunit of the COP9 signalosome, and it functions to regulate NEDD8 attachment to cullins, thereby regulating cullin-RING ligase activity and stability. The biological consequences of CSN5 inhibition might be similar to the inhibition of NEDD8-activating enzyme (BOX 2). AMSH is associated with the endosomal sorting complex required for transport, where its activity regulates trafficking of receptor tyrosine kinases and G protein-coupled receptors[138]. Finally, BRCC36 is part of two complexes, BRCA1 A and BRISC, and has a role in the DNA damage response.

The reasons are incompletely understood but seem to be the result of malfunction or overwhelming of the activities of neuronal 26S proteasomes, autophagy and the endosome–lysosome pathway.

This is shown by experimental genetic findings. *PARKIN* encodes an E3 that ubiquitylates itself and the α-synuclein interacting protein, Synphilin 1. Familial associated mutations in PARK2/Parkin that are defective for E3 activity are linked to autosomal recessive Parkinson's disease[55,56]. Regional ablation of neuronal 26S proteasomes in the brain by deletion of a 26S proteasomal ATPase gene causes the neuropathological hallmarks of Parkinson's disease and dementia with Lewy bodies[57]. Proteasomal dysfunction may be the primary cause of neurodegenerative disease[58]. Genetic deletion of autophagy genes in the brain causes neurodegeneration with the accumulation of deposits of ubiquitylated proteins, although not with the human hallmark neuropathology[59,60]. The degradation of ubiquitylated proteins by the 26S proteasome and autophagy is essential for neuronal homeostasis. When either or both protein-catabolic processes are compromised, neurodegeneration ensues[61,62].

The normal functioning of the endocytic and multivesicular body (MVB) pathway for receptor internalization and degradation is also essential for neuronal homeostasis, as it seems to contribute to normal autophagic activity. Mutations in the endosomal sorting complex required for transport (ESCRT)-III subunit CHMP2B are associated with frontotemporal dementia and amyotrophic lateral sclerosis. Efficient autophagic protein degradation requires functional MVBs. The ESCRT machinery delivers ubiquitylated proteins into invaginations of endosome membranes. The ESCRT machinery then mediates the breaking off of cargo-containing intraluminal vesicles from the perimeter membrane to form MVBs, which may fuse with lysosomes to cause degradation of their protein content. Defects in this pathway would explain the observed neurodegenerative phenotype seen in patients with CHMP2B mutations[63,64].

To extend the cell biological context of neurodegeneration further, it is apparent that the unfolded protein response (UPR) in the endoplasmic reticulum (ER) is connected to autophagy through the key UPR transcription factor XBP1. Genetic XBP1 deficiency causes a significant decrease in the toxicity of mutant SOD1 aggregates (which cause amyotrophic lateral sclerosis) due to an enhanced clearance by autophagy. These data indicate extensive cross-talk between the ER-associated UPR and autophagy to provide protection against neurodegeneration[65]. The malfunction of the UPR in the ER probably triggers autophagy to remove distended ER from the neuron.

Finally, chain-specific protein ubiquitylation has a role in neurodegeneration as K63-linked polyubiquitylation has been detected within pathological lesions of the brains of patients with Alzheimer's, Huntington's and Parkinson's disease. Immunoreactivity to K63 chains is also a feature of inclusions in neurons of proteasome-depleted mice, suggesting a proteasomal contribution to the degradation of K63-linked polyubiquitylated proteins *in vivo* or that K63 polyubiquitylation has a role in inclusion biogenesis[66].

Chronic neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies and amyotrophic lateral sclerosis, are considered to be 'proteinopathies' associated with the intraneuronal accumulation of insoluble protein aggregates in surviving neurons (inclusions) and extracellular amyloid deposits. In Alzheimer's disease, the aggregates contain intraneuronal *tau* protein and extraneuronal

Lewy bodies
Lewy bodies are abnormal protein aggregates that develop inside nerve cells in Parkinson's disease and Alzheimer's disease and some other disorders. They are identified when histology is performed on the brain and appear as spherical masses that displace other cell components.

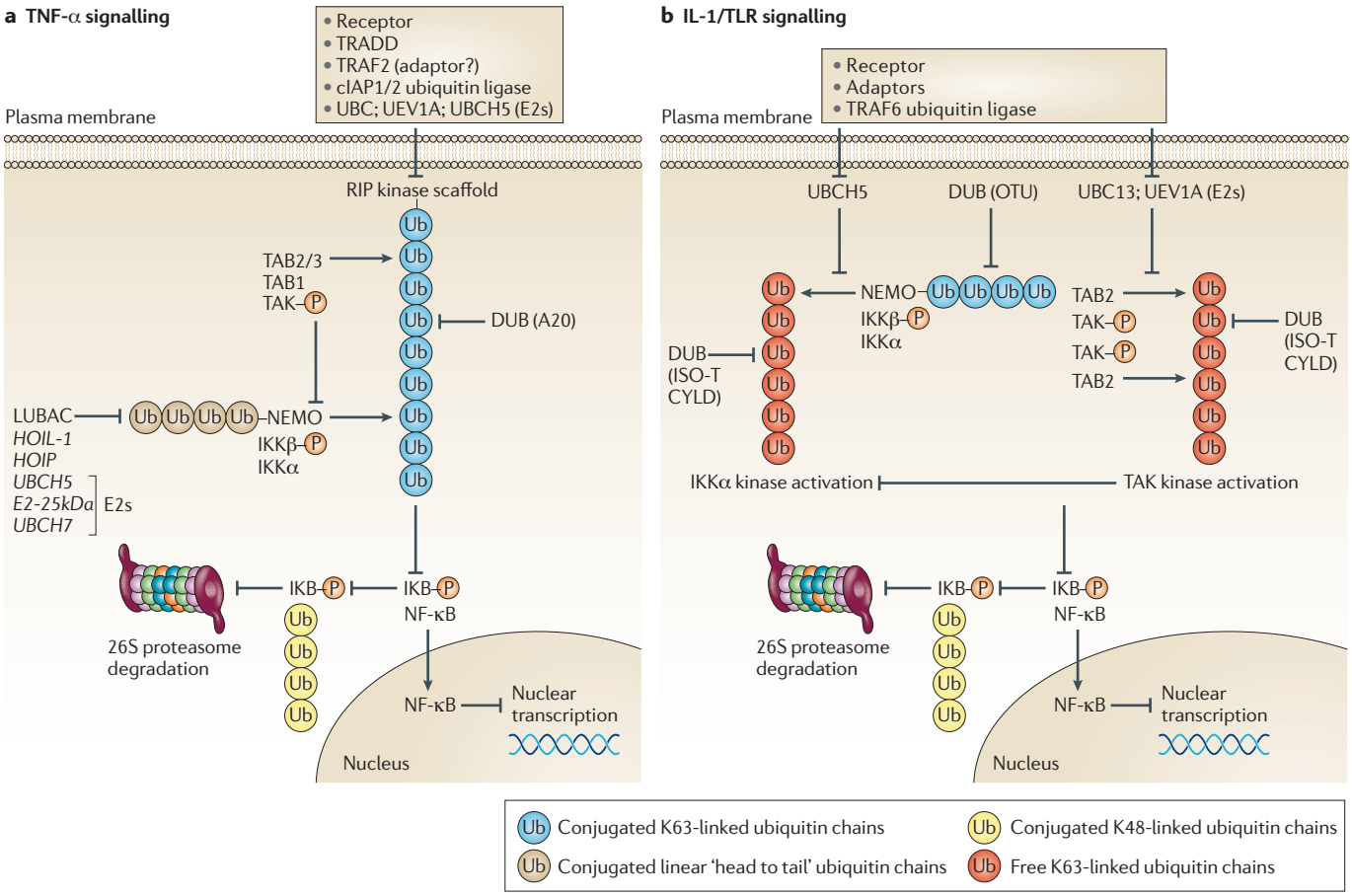

Figure 5 | **Ubiquitin and signalling to nuclear factor- κB (NF-κB). a** | Tumour necrosis factor-α (TNF-α) signalling. **b** | Interleukin-1 (IL-1) receptor 1 (IL1R1) and Toll-like receptor signalling. The diagrams are based on recent reviews[140,141]. Although it is tempting to represent a general consensus, some enzymological steps are controversial due, for example, to enzymological redundancy. The role of ubiquitin (Ub) in signalling to NF-κB needs considerably more investigation. See the main text for more details. DUB, deubiquitylating enzyme; LUBAC, linear ubiquitin chain-assembly complex.

amyloid-β fragments of the Alzheimer precursor protein. In Parkinson's disease and dementia with Lewy bodies, there are intraneuronal deposits of α-synuclein. However, the seminal feature of all these diseases is regional extensive neuronal loss in the brain and spinal cord. It has been difficult to recapitulate these features of the diseases using mouse transgenesis to overexpress amyloidogenic proteins in the brain[67,68]. These approaches have led to intraneuronal aggregates and extraneuronal deposits but not extensive neurodegeneration. By contrast, gene targeting to knock out a proteasomal gene and autophagy genes has resulted in the accumulation of intraneuronal inclusions containing ubiquitylated proteins (some resembling human disease) and extensive regional neuronal loss or neurodegeneration.

Currently, it seems that age-related chronic neurodegenerative disease can be directly attributed to some dysfunction of the UPS and/or autophagy. Any malfunction of these key neuronal processes for regulated removal of proteins results in molecular neuropathological features of neurodegenerative disease. Promotion of the degradation of ubiquitylated proteins by 26S proteasomes (for example, with 26S proteasome activators such as inhibitors of the proteasomal deubiquitylating enzyme USP14 (REF. 69)) or by autophagy (for example, with mTOR (mammalian target of rapamycin) inhibitors[70]) would facilitate slowing or prevention of chronic neurodegenerative disease.

The regional genetic ablation of a proteasomal ATPase gene causes a reduction in neuronal 26S proteasomes, which is a neuropathological feature of both Parkinson's disease and dementia with Lewy bodies, and extensive neuronal death accompanied by some features of neuronal apoptosis. The characterization of the neuronal signal transduction pathways that control cell death in these models by approaches such as analyses of gene expression changes by microarray hybridization and proteomics will delineate these pathways and the downstream consequences of changes in these signalling pathways. Therefore, further studies of the consequences of genetic deletion of neuronal 26S proteasomes (and autophagic functions) will identify receptor and enzyme targets for novel therapeutic developments to slow neuronal death and neurodegeneration. An example of a novel signalling target is the discovery that mutations in optineurin cause amyotrophic lateral sclerosis[71]. Optineurin antagonizes the activity of NEMO by similarly binding to ubiquitin chains. There is a feedback loop with NF-κB increasing

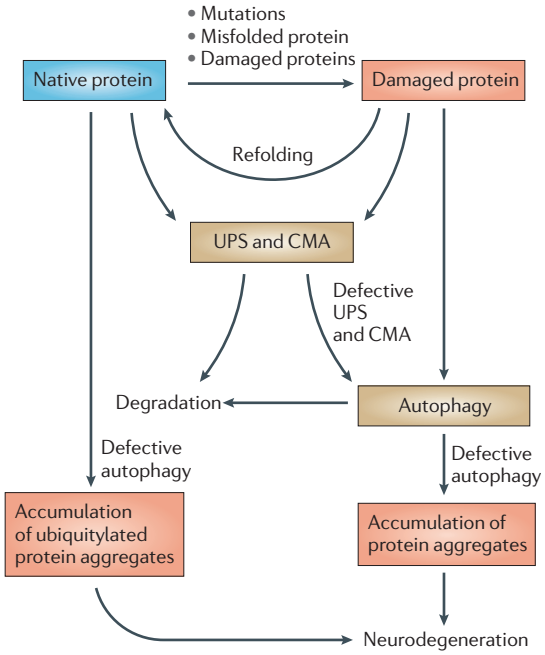

Figure 6 | **Protein quality control and neurodegeneration.** Neuronal proteins are normally degraded by the activities of the ubiquitin–proteasome system (UPS), chaperone-mediated autophagy (CMA) and macro-autophagy (collectively referred to as autophagy), and the endosome–lysosome pathway. Unfolded proteins, proteins altered by mutation or post-translational modifications and proteins that are damaged (for example, by oxidative stress, irradiation or toxins) are recognized by molecular chaperones and delivered to the UPS and autophagy pathways. Age-related and chronic neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies and amyotrophic lateral sclerosis, are 'proteinopathies' associated with the intraneuronal accumulation of insoluble protein aggregates resulting from the malfunction of the neuronal UPS and/or autophagy pathways (see main text for details). The mechanisms of how neurodegeneration results from abnormal protein accumulation due to impaired function of the UPS and autophagy pathways are not known.

the expression of optineurin to inhibit NF-κB activity[72]. Because optineurin mutations prevent this inhibition it is possible that drugs that inhibit NF-κB activity may be useful in the treatment of this debilitating and essentially untreatable disease[71].

Brain tumours. Brain tumours (including aggressive paediatric tumours) are essentially intractable to current therapies without major side effects (for example, developmental disorders). There is therefore a particular need for novel therapeutic advances in this area. As it is clear that the regional targeted ablation of a neuronal proteasomal ATPase gene causes a depletion of neuronal 26S proteasomes and extensive neuronal loss, it is worth considering intervention in the UPS in brain tumours. There are nearly 100 types of brain tumour, many of which develop from glial cells or astrocytes. Anaplastic astrocytoma (also called grade 3 astrocytoma) and glioblastoma multiforme (or grade 4 astrocytoma) are the most common brain tumours in adults and are often found in children. About half of all primary brain tumours are gliomas (astrocytomas, ependymomas and oligodendrogliomas). Current treatments include surgery, radiotherapy and chemotherapy, all with some degree of success but with considerable side effects.

Given that deletion of a proteasomal ATPase gene causes neuronal death[57], inhibitors of proteasomal ATPases (which belong to the AAA (ATPases of alternative activities) superfamily) might be useful for the treatment of brain neuronal tumours and, as all cells contain 26S proteasomes, glial tumours and other tumour cell types too. Such an approach offers a new 26S proteasome target that is independent of inhibiting proteasomal catalytic activities, for which there is already evidence of drug resistance[73]. The anticancer potential of proteasomal ATPase inhibitors might be increased if other cellular ATP-dependent proteases are inhibited. The blood–brain barrier could represent a delivery challenge, but drug-coated 'wafers' incorporating proteasomal ATPase inhibitors might offer neurosurgeons a powerful adjunct to existing drugs for localized chemotherapy[74]. Such proteasomal ATPase inhibitors might also be useful for treating other types of tumour.

Viruses and bacteria. Viruses and bacteria use several strategies to block the UPS and to manipulate specific aspects of the system for infection and replication. As exemplified by the classical example of human papillomavirus encoded E6 oncoprotein, which binds and redirects the E3 ligase E6-AP to target p53 to the proteasome[75], pathogens can be adept at manipulating host-encoded enzymes. Alternatively, pathogen-encoded enzymes that are specific for ubiquitin or UBLs, such as proteases, seem to be a common feature shared by many viruses, bacteria and protozoa. For example, pathogens can code for orthologues of enzymes of the UPS, such as an E2 (REF. 76), or express their own novel analogues of UPS enzymes, such as ubiquitin protein ligases[77]. Some of the proteases share a common origin with mammalian cell-encoded enzymes, but most of them have ancient intrinsic functions, such as processing pathogen protein components, and may have acquired the specificity for ubiquitin or UBLs by interacting with mammalian hosts (and their immune system) throughout evolution.

Many of the pathogen-encoded proteases are different from their mammalian counterparts and are therefore attractive targets for drug development to combat infectious diseases[78]. One example of viral manipulation of the UPS is provided by the Ebola Zaire virus VP35 protein. This viral protein interacts with the transcription factor IRF7, which is required for interferon gene expression, and also interacts with the SUMO E2 enzyme UBC9 and the E3 ligase PIAS1. This interaction increases PIAS1-mediated SUMOylation of IRF7, which represses expression of the interferon gene. By contrast, VP35 does not interfere with the activation of NF-κB, which is required for the induction of many pro-inflammatory cytokines that are needed for viral

infection. These SUMO-related events are part of the mechanism that causes rapid overwhelming infection and eventually pathology such as septic shock[79].

A second example is provided by the HIV virus, which uses MVB proteins to egress from the cell[80] and capitalizes on different stages of the autophagic process to mature. Early, non-degradative stages of autophagy increase HIV yield. HIV Gag-derived proteins co-localize and interact with the autophagy factor LC3 to cause autophagy-promoted productive Gag processing. Later, the HIV protein Nef acts as an anti-autophagic maturation factor through interactions with the autophagy regulatory protein Beclin 1, thereby protecting HIV from degradation[81].

The ISG15 protein was one of the first gene products shown to function as an innate immune protein with broad-spectrum antiviral activity. Protein ubiquitylation might be productive for some RNA viruses but ISGylation is antiviral. A better understanding of the antiviral activities of ISG15 could provide useful insight into the development of novel therapeutic approaches to improve the immune response against such pathogens[82].

Observations of intracellular bacteria, protein ubiquitylation and autophagy have revealed that autophagy of ubiquitylated proteins requires the p62 protein (also known as SQSTM1), which is an adaptor protein with a C-terminal ubiquitin-associated domain for binding to ubiquitylated proteins and an LC3 interaction region for binding the autophagosome protein LC3. Ubiquitylated proteins on the surface of the intracellular bacterium *Salmonella enterica* serovar Typhimurium recruit p62 and cause the autophagic engulfment of the bacteria. This work demonstrates that the detection of bacteria (and almost certainly of misfolded proteins) occurs by a conserved pathway and that p62 has a role in innate immunity[83]. Additionally, the NDP52 protein, not previously known to contribute to innate immunity, recognizes ubiquitin-coated *S.* Typhimurium in human cells (in a similar way to p62) and binds the adaptor proteins Nap1 and Sintbad to recruit Tank-binding kinase 1, a non-canonical member of the IKK kinase family of enzymes. The NDP52 protein also recruits LC3 to activate autophagy against bacteria attempting to colonize the cytoplasm[84].

Bacteria have evolved mechanisms to evade autophagy. For example, the bacterium *Listeria monocytogenes* efficiently escapes autophagy by recruiting the host Arp2/3 complex and Ena/VASP via the bacterial ActA protein to the bacterial surface to disguise the bacteria from ubiquitylation, p62 binding and autophagic sequestration. Significantly, the ability of ActA to mediate protection from ubiquitylation was elegantly demonstrated by generating a GFP–ActA–Q79C chimera, consisting of GFP (green fluorescent protein), the ActA protein and segments of aggregate-prone polyQ (from the Huntington's disease protein). GFP–ActA–Q79C forms aggregates in the host cell cytoplasm. However, these ActA-containing aggregates are not targeted for ubiquitylation and p62 recruitment[85]. One or more steps in these pathways may be subjected to therapeutic intervention to slow or prevent viral and bacterial replication in human cells.

Although few bacteria have *bona fide* proteasomes and none has ubiquitin, one very important bacterial pathogen, *Mycobacterium tuberculosis* (Mtb), does contain a proteolytic system that is analogous and orthologous to the UPS[86]. With estimates of 1.3 million deaths per year attributable to this pathogen[87], there is an urgent unmet medical need for new drugs to target it. Because the prokaryotic ubiquitin-like protein (Pup)-proteasome system of Mtb is essential for pathogenesis[88,89], the various components of the system present excellent opportunities for therapeutic intervention. These include the enzymes involved in 'pupylation' of substrates[90], analogous to the ubiquitin E1–E2–E3 cascade, as well as the 'depupylase' activity that was recently described[91]. The AAA ATPase complex that regulates the Mtb proteasome[92] and the Mtb proteasome itself[88] could also be targeted. Considerable work has already gone into characterizing active site specificity of the Mtb proteasome[93,94] to find inhibitors that could selectively block the function of the Mtb proteasome[95,96] without inhibiting the proteasome of the (human) host.

Future directions and conclusion

The fundamental role of the UPS and UBL conjugation pathways in normal cell function and in disease prompts the search for inhibitors that selectively disrupt pathway function. Despite our limited understanding of the molecular mechanisms of pathway targets, the inhibition of pathway enzymes is an attractive, and increasingly tractable, approach to targeting aberrant signalling pathways in multiple cancers and other diseases. Therapeutic intervention in the UPS, the endosome–lysosome system, the autophagic system and in UBL signalling is an emerging area for the treatment of acute and chronic human diseases, including the treatment of viral and bacterial infections. Some cancers and other disease settings in which these pathways are constitutively active have been shown to be more sensitive to inhibition, potentially limiting the side effects of inhibiting all or part of these key cellular systems.

The precedent for UPS inhibition in cancer has been established by the proteasome inhibitor bortezomib, and second-generation inhibitors are now in clinical development with the aim of improving drug pharmacology. Beyond its use in multiple myeloma and mantle cell lymphoma, the most exciting new therapeutic application of bortezomib is as a drug to prevent antibody-mediated rejection of renal allografts in transplant patients by depleting normal antibody-producing plasma cells[97]. In addition, MLN4924 has established the precedent for inhibiting a specific E1 target, with mechanistic studies indicating the possibility of such an approach for other E1s. Understanding the molecular mechanisms involved in the interactions seen at each step of the UPS and UBL conjugation pathways, between E1s, E2s, E3s, substrates, DUBs and the proteasome itself, is increasing through the use of tools such as small-molecule inhibitors and small interfering RNA, improved compound screening strategies and crystal structure studies. Consequently, there are opportunities present and emerging for other novel therapeutics that target numerous specific pathways in the UPS and UBL conjugation pathways.

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G protein-coupled receptors: novel targets for drug discovery in cancer

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Abstract | G protein-coupled receptors (GPCRs) belong to a superfamily of cell surface signalling proteins that have a pivotal role in many physiological functions and in multiple diseases, including the development of cancer and cancer metastasis. Current drugs that target GPCRs — many of which have excellent therapeutic benefits — are directed towards only a few GPCR members. Therefore, huge efforts are currently underway to develop new GPCR-based drugs, particularly for cancer. We review recent findings that present unexpected opportunities to interfere with major tumorigenic signals by manipulating GPCR-mediated pathways. We also discuss current data regarding novel GPCR targets that may provide promising opportunities for drug discovery in cancer prevention and treatment.

Angiogenesis
The growth of new blood vessels from pre-existing vessels. It is a fundamental step towards an aggressive tumour phenotype.

Metastasis
The ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream and then invade and grow in normal tissues.

Transactivation
Stimulation of gene transcription by transcription factors that can bind to DNA.

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G protein-coupled receptors (GPCRs), which transduce extracellular signals into intracellular effector pathways through the activation of heterotrimeric G proteins (BOXES 1,2), include about 900 members, representing the most prominent family of validated pharmacological targets in biomedicine[1]. As only a small number of these GPCRs are targeted by current drugs, huge efforts are currently being made to exploit the remaining receptors, including approximately 120 members for which no existing ligands have been identified[2]. Both orphan[3–6] and well-characterized GPCRs[7] have been linked to cancer development, starting with the discovery in 1986 of the oncogene *MAS*[8], which encodes a functional GPCR for which the endogenous ligand is ANG1–7 (REF. 9). Moreover, diverse GPCRs were found to be overexpressed in primary and metastatic tumour cells of head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer, breast, prostate and gastric tumours, melanoma and diffused large B cell lymphoma[10] (TABLE 1).

Further evidence confirmed that GPCRs are involved in the initiation and/or progression of cancer. For example, various GPCRs such as chemokines, thrombin, lysophosphatidic acid (LPA), gastrin-releasing peptide, endothelin and prostaglandin receptors have a key role in angiogenesis and metastasis[11–16] as well as in inflammation-associated cancer[7]. In addition, constitutively active GPCRs can be expressed from the genomes of human oncogenic viruses[7].

As more data linking GPCRs to cancer emerge, the pharmacological manipulation of these receptors will become increasingly attractive for the development of novel strategies to target tumour progression and metastasis[7]. In this Review, we discuss the recent advances in our knowledge of the involvement of GPCRs in cancer progression through crosstalk with growth factor receptors, Gα12/13 proteins, LPA-mediated signalling, Hedgehog and WNT transduction pathways, regulation of the apoptotic response and viral factors. Moreover, we address the promising implications of these novel findings towards the discovery of innovative drug targets in cancer prevention and treatment.

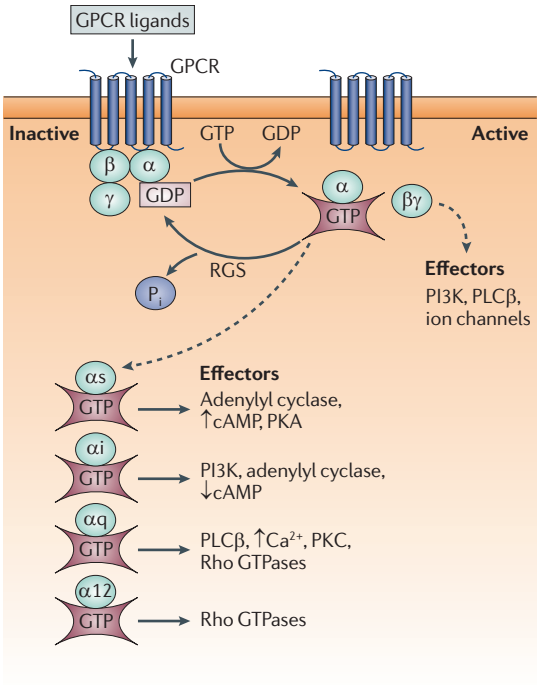
Crosstalk with growth factor receptors

Multilayered crosstalk between GPCRs and growth factor receptors has an instrumental role in orchestrating downstream signalling molecules that are implicated in cancer growth, angiogenesis and metastasis (FIG. 1). Agonist stimulation of diverse GPCRs, such as thrombin, LPA, oestrogen, bombesin and endothelin, can lead to transactivation of epidermal growth factor (EGF) receptor (EGFR) through autocrine and paracrine release of EGF-like ligands tethered at the cell surface and the subsequent generation of intracellular signalling that promotes cancer progression[17–19] (FIG. 2). Indeed, the functional crosstalk between GPCRs and EGFR contributes to the progression of colon, lung, breast, ovarian, prostate and head and neck tumours[18,20]. Therefore, increased understanding of the specific signalling pathways involved in EGFR transactivation by GPCRs[21] will facilitate drug discovery for novel pharmacological approaches in cancer patients.

GPCR-mediated activation of EGFR signalling. EGFR is a crucial component of the signal transduction pathways that regulate tumour growth, survival, migration

Box 1 | **G proteins: classification and mechanism of action**

G protein-coupled receptors (GPCRs) are targets of a wide variety of ligands and are implicated in many pathophysiological functions. A common molecular architecture consisting of seven transmembrane domains connected by three intracellular and three extracellular loops characterizes all GPCRs. Their activation triggers the signalling that is mediated by their membrane-bound partners, the heterotrimeric G proteins, which regulate a broad range of biological processes[211]. The G proteins, which in their inactive state consist of a Gβγ monomer and a guanine diphosphate-bound Gα subunit, belong to four families — Gαs, Gαi, Gαq/11 and Gα12/13 — on the basis of the α subunit sequence identity and signalling activity. Following ligand activation, GPCRs catalyse the exchange of GDP for GTP on the Gα subunit, leading to a decreased affinity of Gα for Gβγ. The resulting dissociation of the heterotrimer allows the GTP-bound Gα and free Gβγ to interact with several downstream effectors, including adenylyl cyclases, phosphodiesterases, phospholipases, tyrosine kinases and ion channels[211]. RGS proteins accelerate the GTPase activity of Gα subunits and the subsequent deactivation of G proteins (see Box 2). cAMP, cyclic AMP; PI3K, phosphoinositide 3-kinase; PKA and PKC, protein kinase A and C; PLCβ, phospholipase Cβ; Rho, RAS homology; RGS, regulator of G protein signalling.



and resistance to chemotherapies in various human malignancies[22]. In this respect, the intensity and duration of EGFR transactivation — through the activation of various extracellular stimuli — determines differences in responses in cancer cells that are linked to malignant tumour phenotype and poorer patient outcome[20–22].

Inter-receptor crosstalk between GPCRs and EGFR occurs in both transformed and nontransformed cells[18,20]. For instance, the transactivation of EGFR by agonist-activated GPCRs has a key role in regulating many important biological processes, such as fertilization, angiogenesis, neurogenesis, heart development and tumour progression, and typically requires growth factor cleavage mediated by matrix metalloproteinases (MMPs)[23–25]. In particular, LPA, the acetylcholine receptor agonist carbachol and the inflammatory molecules prostaglandin E2 (PGE2) and bradykinin stimulate the growth and invasion of HNSCC cells by triggering functional crosstalk between their cognate GPCRs and EGFR-mediated signalling[24,26] (FIG. 1). The CXC chemokine CXCL12 (also known as stromal cell-derived factor 1α (SDF1α)), which is a growth regulator and stimulator of angiogenic factors in cancer cells, is an additional example of a GPCR ligand that can induce the EGFR transduction cascade, this time through the activation of the cognate GPCR CXCR4 (REFS 27,28) (FIG. 1). The GPCR protease-activated receptor 1 (PAR1) has a functional role in the growth and metastasis of different types of tumour, including breast cancer[29–32]. The proteolytic activation of PAR1 by thrombin, which activates EGFR signalling, induced cellular invasion and tumour growth *in vivo*, as assessed by mammary fat pad xenografts[33].

Preliminary experimental and clinical data show that interfering with endothelins and cognate GPCRs could be a new mechanism-based antitumour strategy, given the involvement of the endothelin axis in cancer development and progression[34]. In particular, the endothelin A subtype receptor (ET_AR) mediates endothelin 1 (ET1)-induced proliferation of cancer cells, angiogenesis and epithelial-to-mesenchymal transition[34]. ET1 cooperates with EGFR signalling in stimulating cancer cells, as shown by the ET_AR antagonist ZD4054 used in combination with the EGFR inhibitor gefitinib, which strongly reduces the proliferation and invasion of ovarian cancer cells[35]. Moreover, in rhabdomyosarcoma cells, ZD4054 used alone reversed ET1-induced phosphorylation of focal adhesion kinase and paxillin, leading to the inhibition of the invasive phenotype mediated by these adhesion factors[36]. ZD4054 also has inhibitory activity in breast cancer cells[37] and in animals bearing ovarian tumour xenografts[36]. Several clinical trials are underway to test the anticancer efficacy of ZD4054 either administered alone (ClinicalTrials.gov identifiers: NCT01119118, NCT00626548, NCT00554229, NCT01000948, NCT00997945, NCT00090363, NCT01134497, NCT01168141 and NCT00055471) or in combination with cytotoxic drugs (ClinicalTrials.gov identifiers: NCT00929162, NCT00745875, NCT00617669, NCT00314782 and NCT01205711).

These investigations, in particular the *in vivo* studies, showed that ET_AR is a promising target for novel therapeutic strategies in patients with cancer. In addition, the potent selective ET_AR antagonist atrasentan showed beneficial effects in a Phase III trial in patients with hormone-refractory prostate cancer, although this

Transformed cells
Cells that acquire the properties and behaviour of cancer cells.

Nontransformed cells
Cells that have regular features.

Epithelial-to-mesenchymal transition
A process characterized by the loss of cell adhesion and increased cell mobility, which can occur during embryonic development and in the malignant transformation of cells.

Focal adhesion kinase
A non-receptor tyrosine kinase that resides at the sites of integrin clustering, which are known as focal adhesions. It plays an important part in cell proliferation and migration, as well as in tumour growth, invasion and survival.

Paxillin
A focal adhesion-associated adaptor protein that facilitates the assembly of multi-protein complexes involved in the activation of signalling pathways that lead to cell migration and survival.

Amphiregulin
A member of the epidermal growth factor family that interacts with the epidermal growth factor receptor to regulate the growth of normal and transformed cells.

Cyclin D1
A member of the cyclin family that is required for cell cycle G1/S transition. Mutations, amplification and overexpression of cyclin D1 contribute to tumorigenesis and are observed frequently in various tumours.

Stroma
The supportive framework of an organ, gland or other structure, usually composed of connective tissue.

trial did not meet its primary end point — defined as the onset of metastases — owing to large geographical differences in the time of disease progression[38]. The overall antitumour effects of atrasentan were confirmed using a mouse model of prostate cancer metastatic colonization; however, the survival benefit depended on the stages of bone metastasis[39]. Whether atrasentan in combination with cytotoxic drugs would be more effective in treating cancer patients is under evaluation in clinical trials (ClinicalTrials.gov identifiers: NCT00653328 and NCT00134056). The complex part played by endothelin signalling, particularly in the initial bone colonization, requires valuable tools to better assess the pharmacological manipulation according to the clinical conditions of cancer patients.

Given the stimulatory function elicited by ET_AR and human EGFR2 (HER2) in certain tumours, atrasentan was also used in combination with the monoclonal HER2-specific antibody trastuzumab. The inhibition of basal and EGF-induced proliferation and invasion of breast cancer cells was superior when targeting both receptors compared with each agent used alone[40]. Further studies in animal models would support the hypothesis that interfering with both HER2 and ET_AR signals is an effective approach in patients with breast cancer.

GPCR-induced growth factor shedding. The transactivation of EGFR mediated by members of the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent proteases is relevant to the development and progression of diverse types of human tumour[41]. Among the MMPs that are specialized in releasing membrane-tethered proteins, the tumour necrosis factor-α (TNFα) converting enzyme (TACE; also known as ADAM17) has received much attention owing to its ability to activate growth factor receptors by causing the shedding of their extracellular domain from the surface of different cancer cells[42]. In this context, it is noteworthy that LPA, sphingosine 1-phosphate (S1P) and thrombin also activate the EGFR mitogenic signalling through TACE and ADAM15 in breast cancer cells[20]. Although TACE inhibitors were entered into clinical trials, their peptidic and peptide-like chemical structures afforded low bioavailability and major pharmacokinetic problems, limiting their clinical effectiveness. Therefore, novel orally bioavailable inhibitors of TACE have been developed[43], providing the first step towards the identification of new, highly desirable anticancer compounds.

Overall, the whole inhibition of the EGFR axis (that is, targeting EGFR ligand cleavage, ligand uptake, receptor phosphorylation and activation (FIG. 2)), might be more effective than the inhibition of a single biological event. In this regard, the blockade of TACE prevented the shedding of both transforming growth factor-α and amphiregulin, abolishing the subsequent EGFR-dependent stimulation of breast cancer cells[44]. Using a TACE inhibitor in combination with an EGFR blocking agent, suboptimal doses of these drugs showed enhanced growth inhibition and increased apoptosis with respect to the effects obtained by each compound used alone[45].

GPCR-mediated activation of EGFR by hormones. 17β-Estradiol (E2) binds to and activates oestrogen receptor (ER)α and β, which in turn regulate the transcription of genes involved in numerous physiological functions[46–47]. In addition, E2 induces rapid non-genomic effects through the G protein oestrogen receptor (GPER; also known as G protein-coupled receptor 30 (GPR30)) in normal and cancer cells[5,48–52]. The GPER-activated signalling is clearly distinct from that of the classical nuclear ERs; however, the two transduction pathways might interact cooperatively to stimulate relevant biological responses in some cases[50,53,54]. As observed for other EGFR activators, oestrogens induce GPER-mediated signalling through metalloproteinase activity and release of heparan-bound EGF, which then leads to EGFR transactivation[18] (FIGS 1,2). Moreover, a recent microarray analysis showed that E2 and 4-hydroxytamoxifen — an ER inhibitor but a GPER activator — induced the rapid GPER-mediated transcription of genes, such as MMPs and effectors of growth factor activity, that are involved in cancer progression[55]. Triggering a positive loop, ligand-induced activation of EGFR led to the upregulation of GPER expression, which further contributed to the stimulatory action elicited by E2 in tumour cells[54,56].

E2, acting through GPER, also induced gene transcription and the migration of breast carcinoma-associated fibroblasts[57], which strongly contribute to cancer development and progression[58]. In addition, E2 induced the physical interaction between GPER and phosphorylated EGFR and the recruitment of both receptors to the cyclin D1 promoter region, suggesting that GPER might act as a transcription factor in carcinoma-associated fibroblasts[57]. Such observations further support the concept that the complex interactions between cancer cells and stroma are attractive drug targets[59–60]. In patients with breast, endometrial and ovarian carcinomas, GPER overexpression was associated with negative clinical features and poor survival rates[61–63], suggesting that new pharmacological tools are required to inhibit the stimulatory effects elicited by oestrogens, including those mediated through GPER, in cancer and/or stroma-derived cells.

Box 2 | **RGS proteins in cancer**

The family of regulator of G protein signalling (RGS) proteins all contain an RGS domain, which through its GTPase-activating action accelerates the deactivation of heterotrimeric G proteins, leading to the attenuation or termination of G protein-coupled receptor (GPCR)-mediated pathways. RGS proteins are involved in diverse types of cancer[212]. For example, the ability of RGS proteins to attenuate or terminate lysophosphatidic acid-dependent G protein-mediated signalling leads to the inhibition of cancer cell proliferation and migration[213]. In addition, the RGS family member RGS5 has a pivotal role in vessel remodelling during carcinogenesis[214]. Accordingly, tumours arising in a RGS5-deficient background display vessel morphological changes and improved blood flow, which allows enhanced lymphocyte access within the tumours; anticancer immunotherapy had beneficial effects[214]. Moreover, mutations in RGS genes confer a reduced risk to develop some types of cancer, including lung and bladder tumours[212]. Further *in vivo* studies are required to better define the specific role played by RGS proteins in cancer progression as well as to highlight the beneficial effects deriving from the potential manipulation of RGS proteins.

Interaction between GPCRs and insulin or insulin-like growth factor I receptors. The crosstalk between GPCRs and insulin or insulin-like growth factor I (IGFI) receptor (IGFIR) has a crucial role in the regulation of many physiological functions as well as in the development of diverse malignancies[64]. For example, exposure of BxPc3, HPAF II and PANC1 ductal pancreatic adenocarcinoma cells to insulin rapidly enhances inositol 1,4,5-trisphosphate and intracellular Ca^{2+} levels induced by multiple Gαq-coupled GPCR agonists, such as ANG II, bradykinin, bombesin, neurotensin and vasopressin[65]. This potentiation of GPCR activity involves the PI3K–AKT–mTOR transduction pathway[65], which is a key player in mediating many stimulatory effects induced by insulin and IGFI[66].

It is noteworthy that metformin (1,1-dimethyl biguanide hydrochloride), one of the most widely prescribed drugs for the treatment of type 2 diabetes, activates AMP kinase (AMPK), which in turn blocks mTOR complex 1 activity, leading to the inhibition of cancer cell growth[64,67,–68]. Moreover, metformin acting through AMPK prevents the insulin-dependent increase in calcium signalling, DNA synthesis and anchorage independent proliferation induced by GPCR agonists in pancreatic cancer cells[69]. Recent findings also provide strong motivation for the clinical development of metformin as a non-toxic therapeutic that can interdict the breast cancer stem cell phenotype by targeting the epithelial-to-mesenchymal transition, which has a central role in the ontogenesis of the molecular signature of breast cancer stem cells[70]. Overall, the capability of metformin to inhibit the functional crosstalk between insulin–IGFIR and GPCR signalling may be beneficial for the prevention and therapy of certain types of cancer. This is in agreement with previous epidemiological studies that showed a positive relationship between the administration of metformin and a reduced risk of pancreatic, breast and prostate tumours in patients with diabetes[64,71].

Given the major EGFR and the IGFIR contribution to tumour development and progression, EGFR-targeting monoclonal antibodies and tyrosine kinase inhibitors are currently used for the treatment of several types of tumour[72]. However, primary resistance and acquired resistance to these agents often limit the clinical efficacy of mono-specific targeted therapy. Results from *in vitro* and *in vivo* studies indicate that crosstalk between EGFR and IGFIR, even through common interaction partners such as GPCRs, can lead to acquired resistance against EGFR-targeted drugs[72]. The involvement of this multilayered cooperation in chemoresistance provides clear bases for targeting EGFR and IGFIR in combination with GPCRs as a novel therapeutic approach[72].

Table 1 | **Selected G protein-coupled receptors that are involved in cancer**

Ligand	Receptor	Cancer
Lysophosphatidic acid	Lysophosphatidic acid receptors	• Colon cancer [97] • Ovarian cancer [75,95,99,157] • Prostate cancer[7] • HNSCC[26] • Breast cancer[96,98]
Sphingosine 1-phosphate	Sphingosine 1-phosphate receptors	• Glioma[84–86] • Breast and prostate cancers[83] • Ovarian cancer[96]
Thrombin	Protease-activated receptor 1	• Breast cancer[35,74] • Colon cancer[32] • HNSCC[31] • Prostate cancer[33]
Gastrin-releasing peptide	Gastrin-releasing peptide receptor	• HNSCC[13] • Lung and pancreatic cancers[18] • Prostate cancer[14]
Endothelin 1	Endothelin receptors	• Ovarian cancer[37] • Colon and prostate cancers[36] • Breast cancer[39,42] • Endometrial cancer[36] • Rhabdomyosarcoma[38]
Prostaglandin E2	Prostaglandin E2 receptors	• HNSCC[26] • Breast cancer[7] • Colon cancer[16] • Lung cancer[7] • Prostate cancer[7,14]
Bradykinin	Bradykinin receptors	• Chondrosarcoma[176] • HNSCC[26] • Prostate cancer[14]
Chemokine (CXC motif) ligand 12 (also known as stromal cell-derived factor 1)	CXC chemokine receptor 4	• Melanoma[29,88] • Pancreatic cancer[29] • Prostate cancer[73] • Breast cancer[30,73,87] • Ovarian and thyroid cancers[29] • HNSCC[7] • Lung cancer[73] • Neuroblastoma and kidney cancer[29]
Angiotensin II	Angiotensin II type 1 receptor	• Gastric cancer[175] • Prostate cancer[7]
Interleukin-8	CXC chemokine receptor 2	• HNSCC, lung cancer and melanoma[7]
Oestrogen and anti-oestrogen	G protein-coupled oestrogen receptor	• Breast cancer[56–59] • Ovarian cancer[52] • Thyroid cancer[51] • Endometrial cancer[50,56] • Prostate cancer[54]
Orexins	Orexin receptor 1	• Neuroblastoma, colon and pancreatic cancers[166]
Hedgehog ligands	Smoothened	• Basal cell carcinoma and medulloblastoma[129] • Prostate, gastrointestinal, haematological cancers and glioma[112]
WNT ligands	Frizzled	• Colon cancer[142,145] • Lung cancer[4,139] • Breast, gastric and thyroid cancers and melanoma[139]

HNSCC, head and neck squamous cell carcinoma.

Gα12/13-coupled receptors

Members of the Gα12/13 subfamily of heterotrimeric G proteins show transforming activity in model systems, including nude mice which were injected with fibroblasts previously transfected with the cloned α subunit of mouse Gα12 protein[73,74]. Moreover, the activation of Gα12/13-dependent signalling by circulating or locally produced agonists contributes to cancer development and progression[7]. For instance, the Gα12/13 proteins are involved in the migration of cancer cells and angiogenesis stimulated by either LPA[75,76] or activated receptor tyrosine kinases such as EGFR, platelet-derived growth factor receptor and vascular endothelial growth factor receptor (VEGFR)[77]. In addition, the Gα12/13-coupled PAR1 mediates cell invasion and angiogenesis induced

by thrombin[31,78]. The ability of the Gα12/13 proteins to negatively regulate the adhesive functions of cadherins, a family of integral membrane proteins that mediate cell–cell adhesion, represent a further mechanism through which this G protein subfamily might be involved in the migration and invasion of cancer cells[79].

Many of the effects of Gα12/13 signalling are mediated by Rho (RAS homology) family members[80,81] (FIG. 1) — small GTPases that are highly conserved across species[82] — which contribute to diverse cellular processes involved in cancer progression, including cytoskeletal dynamics, cell cycle progression, transcriptional regulation, cell survival and vesicle trafficking. The oncogenic action of various Rho GTPases correlates with their increased expression and activity in various cancers[83]. In particular, the GPCR ligands S1P, LPA, thrombin, PGE2 and thromboxane A2 trigger RhoA signalling through activation of their cognate Gα12/13-linked receptors[84,85]. For instance, S1P regulates glioma cell motility depending on the expression pattern of S1P1/2 receptors[86–88]. Furthermore, CXCL12 activates CXCR4, which is highly expressed in frequently metastatic sites[89,90] and is transcriptionally regulated by hypoxia-inducible factor 1α (HIF1α) stimulation that occurs in the hypoxic tumour microenvironment[91]. It is worth mentioning that CXCL12-dependent RhoA activation is involved in the invasion of melanoma cells, whereas the expression of a GTPase-deficient Gα13 form impairs chemokine-induced invasion of melanoma cells and metastasis observed *in vivo*[92,93].

The aforementioned GPCR ligands, such as thrombin, LPA and S1P, through coupling of their cognate receptors to Gα12/13 proteins, stimulate angiogenic responses directly and/or through the involvement of angiogenic mediators such as VEGF[7]. In addition, thrombin contributes to tumour cell migration, nodule growth and angiogenesis, also inducing the transcription of genes such as *twist* and *cathepsin D* that are involved in the upregulation of vascular growth factors and receptors[94–96]. On the basis of these findings and data regarding the involvement of the Gα12/13-coupled receptors, such as PAR1, LPA and S1P receptors, in tumour growth, metastasis and angiogenesis, further studies are required to identify anticancer drugs targeting Gα12/13 proteins and/or Rho-mediated signalling *in vivo*.

LPA signalling in migration and invasion

The bioactive phospholipid LPA signals through at least six receptors (LPA1–LPA6) that belong to the GPCR family. LPA1–LPA3 (also known as EDG2, EDG4 and EDG7, respectively) are expressed by endothelial differentiation genes[97], whereas the deorphaned receptors LPA4 (also known as GPR23/P2Y9), LPA5 (GPR92) and LPA6 (GPR87) are included within the purinergic family of GPCRs[98–100]. The biological activity exerted by LPA contributes to cancer cell proliferation, invasion, migration and angiogenesis[97,101,102]. For instance, an elevated production of LPA and/or an aberrant expression of its receptors show potent mitogenic capabilities leading to cancer initiation and progression[103,104]. In particular, studies in breast[105] and ovarian tumour cells[75,106] demonstrate that LPA can induce cell migration through the activation of the Rho-dependent transduction pathway. Collectively, these results indicate that LPA is an important player in cancer development and so blocking LPA activity could be a promising drug target.

Taking into account the aforementioned findings, bioactive LPA analogues have been developed that act as receptor antagonists and are resistant to phosphatase, acyltransferase and lipase activities[107]. For instance, stable LPA analogues include the α-hydroxymethylene[108] and α-fluoromethylene phosphonates[109]. In addition to the manipulation of LPA activity through cognate receptors, the biosynthesis of LPA is considered a valid target for therapeutic purposes[110]. LPA is generated from lysophosphatidylcholine by the lysophospholipase D activity of the tumour-associated protein autotaxin[110]. Autotaxin, which is strongly upregulated in invasive tumours and is implicated in cancer invasion and metastasis[111], is feedback-inhibited by LPA[112]. Therefore, an ideal anticancer drug which targets the LPA cascade should simultaneously abrogate the signalling mediated by LPA GPCRs and abrogate the production of LPA by interfering with autotaxin activity[113]. In this regard, the palmitoyl α-bromomethylenephosphonate-1 (BrP–LPA) shows a pan-antagonism for LPA1–4 as well as having the capacity to inhibit autotaxin, leading to the repression of cell migration and invasion[108]. Moreover, in a lung cancer xenograft model, treatment with BrP–LPA diastereomers results in the inhibition of tumour growth and angiogenesis[76].

A structure–function study of carbacyclic analogues of cyclophosphatidic acid demonstrates that these compounds act as selective inhibitors of autotaxin without agonism of LPA1–3. In particular, cyclophosphatidic acid inhibits cancer cell invasion *in vitro* and suppresses the metastatic ability of melanoma cells *in vivo*[114]. In a further study, the migration of melanoma cells was also reduced using different autotaxin inhibitors[115]. Recently, stereoisomers of BrP–LPA inhibited autotaxin and antagonized five LPA receptors, thereby preventing the migration and invasion of breast cancer cells[116]. Analogously, these compounds induced breast tumour regression in xenograft models[116].

Hedgehog and WNT transduction pathways

The Hedgehog (HH) transduction pathway has a crucial role during embryo development[117]. In adult tissues, the HH pathway contributes to stem cell maintenance, tissue repair and regeneration, but it is also implicated in the development of basal cell carcinoma, a subset of medulloblastoma and other sporadic malignancies, including those in the pancreas, prostate, lung and breast, as confirmed using experimental mouse models[118]. The 12-transmembrane receptor called Patched (PTC) and the 7-transmembrane GPCR named Smoothened (SMO), are key mediators of the HH transduction cascade[119]. In the absence of secreted HH ligands, such as Sonic HH (SHH), PTC inhibits the activity of SMO, whereas ligand binding to PTC releases SMO inhibition[120,121] (FIG. 1). Activated SMO mediates HH signalling leading to the stimulation of the glioma-associated

Anchorage independent proliferation
The proliferation of cells that are unattached to a substrate. Such proliferation can occur in cancer cells and contribute to metastasis.

Primary resistance
Lack of efficacy of a drug (or drugs) in patients who had never received treatment with that drug (or drugs).

Acquired resistance
A lack of efficacy of a drug (or drugs) that initially elicited a response.

Endothelial differentiation genes
Genes that encode G protein-coupled receptors for lysophosphatidic acid or the lysophospholipid mediator sphingosine 1-phosphate.

Diastereomer
An isomer that is a stereoisomer of a compound with two or more chiral centres and that is not a mirror image of another stereoisomer of the same compound.

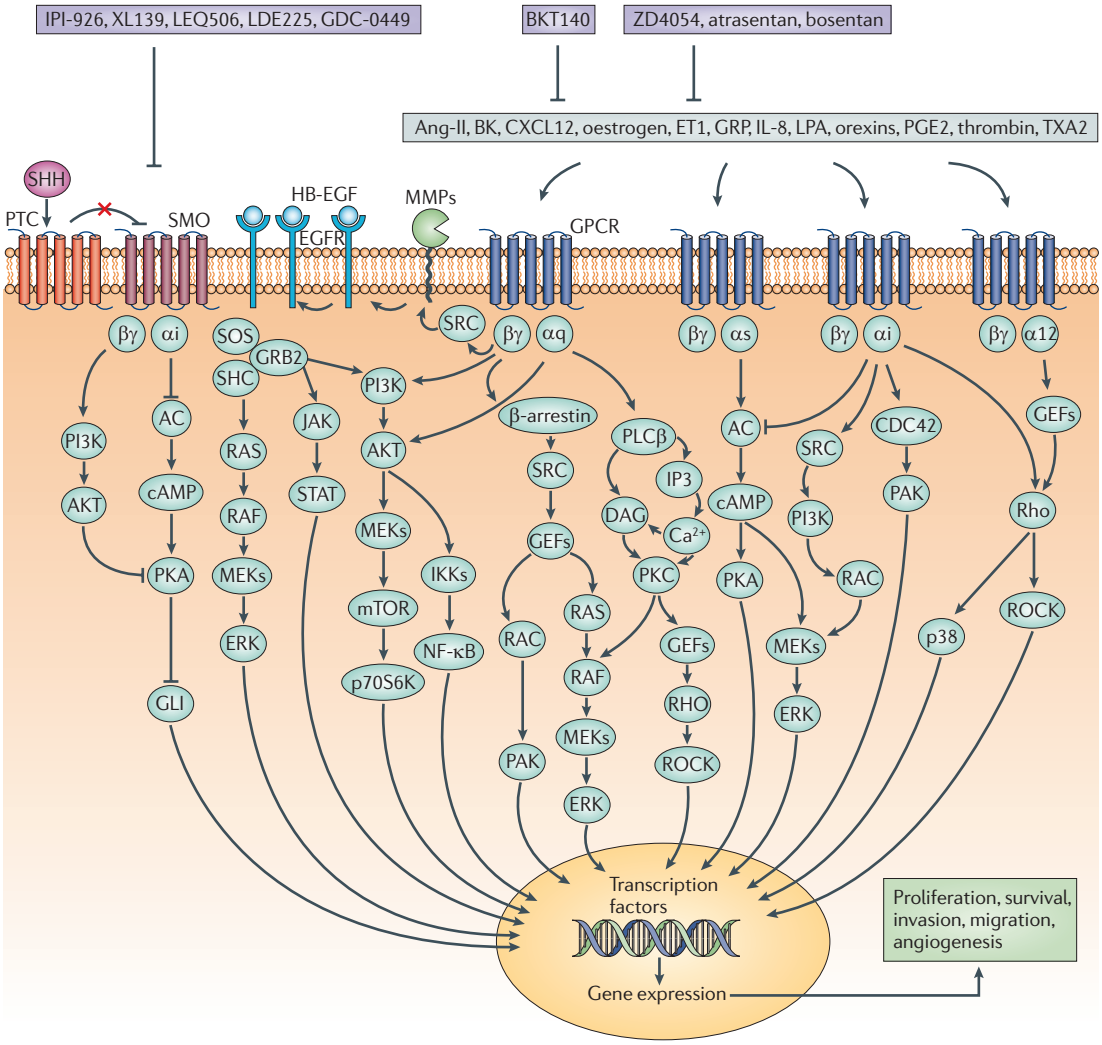


Figure 1 | **Selected transduction pathways involved in GPCR-mediated cancer signalling.** Many G protein-coupled receptor (GPCR) ligands activate several downstream effectors that stimulate cancer progression and invasion. A single agonist-activated GPCR can couple to one or more families of G proteins, and each G protein triggers diverse transduction pathways. Gs proteins stimulate AC and increase cAMP levels, promoting the activation of PKA- and ERK-dependent signalling. Gαi proteins inhibit AC and activate SRC, PI3K, RAC–MEK–ERK and CDC42–PAK and Rho-mediated signals. Gαq proteins activate PLC, which cleaves phosphatidylinositol-4,5-bisphosphate (PIP2; also known as PtdIns(4,5)P_2) into DAG and IP3, leading to calcium mobilization. Moreover, PKC activation triggers RhoGEFs and MAPK cascades. In addition, Gαq activates AKT signalling which modulates mTOR and NF-κB transduction pathways. Gα12/13 proteins activate RhoGEFs which initiate Rho-dependent signalling through ROCK and p38 MAPK. The Gβγ dimers modulate the activity of the downstream effectors RAS and RAC and trigger AKT-mediated signals. Crosstalk between GPCRs and growth factor receptors, such as EGFR, regulates many intracellular pathways, including ERK, JAK–STAT and PI3K. The 12-transmembrane receptor PTC and the 7-transmembrane receptor SMO, which acts as a GPCR, are key mediators of the Hedgehog (HH) transduction cascade. The binding of the HH ligand SHH to PTC activates SMO, which leads to GLI stimulation that inhibits AC through Gαi proteins as well as activating PI3K–AKT signalling by Gβγ. Ligands and inhibitors (grey boxes) that can regulate GPCRs signalling are shown. The exact pathways activated are dependent on cell type. AC, adenylyl cyclase; Ang-II, angiotensin-II; BK, bradykinin; cAMP, cyclic AMP; CDC42, cell division cycle 42; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; ET1, endothelin 1; GEFs, guanine nucleotide exchange factors; GLI, glioma-associated oncogene homologue; GRP, gastrin-releasing peptide; GRB2, growth factor receptor-bound protein 2; HB-EGF, heparan-bound EGF; IKKs, IκB kinases; IL-8, interleukin-8; IP3, inositol triphosphate; JAK, Janus kinase; LPA, lysophosphatidic acid; MEK, MAP kinase–ERK kinase; MMPs, metalloproteinases; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; p70S6K, ribosomal protein S6 kinase; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PKA and PKC, protein kinase A and C; PAK, p21/CDC42/RAC1-activated kinase; PLCβ, phospholipase Cβ; PTC, Patched; Rho, RAS homology; ROCK, Rho-associated coiled-coil containing protein kinase; SHC, SHC-adaptor protein; SHH, Sonic hedgehog; SMO, Smoothened; SOS, son of sevenless; STAT, signal transducer and activator of transcription protein; TXA2, thromboxane A2.

oncogene homologue (GLI) transcription factors (GLI1, GLI2 and GLI3), which are involved in proliferative and anti-apoptotic effects in diverse tumours[120]. GLI1 expression, considered the most reliable marker of the HH activity, is upregulated in various malignancies[122].

Targeting HH signalling. As far as HH inhibitors are concerned, cyclopamine suppresses cell growth and induces apoptosis either alone or in combination with other drugs in cancer cells and xenograft models[123,124]. Given the acid lability and the poor water solubility of cyclopamine as an administrable drug, numerous derivatives with a better bioavailability and a higher biological potency have been developed. For example, the SMO inhibitor KAAD-cyclopamine prevented the proliferation of pancreatic tumour cells[125] and attenuated the motility and invasiveness of hepatocarcinoma and gastric cancer cells[126,127]. Furthermore, KAAD-cyclopamine used in combination with the TNF-related apoptosis-inducing ligand (TRAIL) induced rapid apoptotic effects in TRAIL-resistant glioma cells[128].

The orally bioavailable HH inhibitor IPI-269609 (REF. 129) abrogated the migration and colony formation of pancreatic cancer cells and inhibited pancreatic cancer cell metastases in orthotopic xenografs[130]. The HH inhibitor IPI-926, which exhibited antitumour activity in ovarian cancer animal models[131], is currently in Phase I clinical trials in patients with advanced and/or metastatic solid tumours (ClinicalTrials.gov identifier: NCT00761696; TABLE 2). IPI-926 might also be a useful combination therapy in other forms of cancer, as in a genetic mouse model of pancreatic tumours it elicited beneficial effects when used together with gemcitabine[132].

Recently, new SMO antagonists showed antitumour activity acting as HH inhibitors in melanoma, in basal cell carcinoma and in a genetic mouse model of medulloblastoma[133–135]. The SMO antagonist BMS-833923 (also known as XL139) is currently under evaluation in Phase I clinical trials in patients with basal cell carcinoma, small cell lung carcinoma, stomach and oesophageal tumours, multiple myeloma and leukaemia (Clinical Trials.gov identifiers: NCT00670189, NCT00884546 and NCT01218477). In addition, the HH antagonist GDC-0449, which produced promising antitumour responses in early clinical studies of tumours driven by mutations in the HH pathway[135], was entered into clinical trials for the treatment of diverse types of cancer (ClinicalTrials.gov identifiers: NCT00959647, NCT01163084, NCT01154452, NCT01071564, NCT00939484, NCT00739661, NCT00636610, NCT00607724, NCT01209143, NCT00822458, NCT01239316, NCT00968981, NCT00980343, NCT01096732, NCT00833417, NCT01174264, NCT00957229, NCT01160250 and NCT01201915; TABLE 2). However, in a patient enrolled in the GDC-0449 Phase I study, the metastatic medulloblastoma relapsed after an initially rapid but incomplete response[136]. An acquired somatic missense mutation in SMO, which did not affect HH signalling but prevented GDC-0449 binding to SMO, caused this drug insensitivity[137]. Therefore, SMO mutations can be considered as a potential mechanism responsible for drug resistance in cases of HH pathway-dependent tumours relapse.

μNeoplastic tissue
An abnormal mass of tissue that is generated by uncoordinated and excessive cell proliferation that persists after cessation of the stimuli.

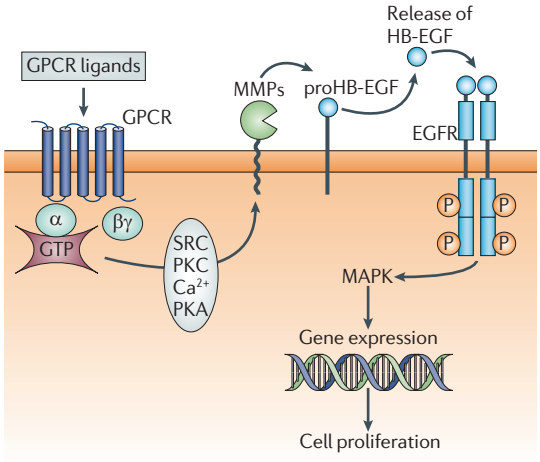


Figure 2 | **GPCR-induced transactivation of EGFR.** Ligand-activated G protein-coupled receptors (GPCRs) induce the transactivation of epidermal growth factor receptor (EGFR) through several mediators, including SRC kinases, Ca^{2+}, protein kinase C (PKC) and PKA. The generation of heparan-bound epidermal growth factor (HB-EGF), which occurs by matrix metalloproteinase (MMP)-mediated cleavage of proHB-EGF, is required for the activation of the EGFR–mitogen-activated protein kinase (MAPK) transduction pathway, which is largely involved in gene expression and cell proliferation.

The use of GLI inhibitors such as GANT58 and GANT61, which were capable of preventing tumour growth *in vitro* and in xenograft cancer models[138], could be an alternative route in patients who experienced the failure of treatment with SMO antagonists. Furthermore, the clinical anticancer benefits of the SMO inhibitors IPI-926 (ClinicalTrials.gov identifier: NCT01130142), BMS-833923 (ClinicalTrials.gov identifiers: NCT00927875 and NCT00909402) and GDC-0449 (ClinicalTrials.gov identifiers: NCT00878163, NCT00982592, NCT01064622, NCT01143415, NCT00887159, NCT01088815 and NCT01195415) administered in combination with cytotoxic drugs are currently under evaluation in patients with diverse types of metastatic or inoperable tumours.

Targeting WNT signalling. Like the HH cascade, the canonical WNT–β-catenin signalling contributes to the coordination of developmental transitions as well as to tumour formation[139]. Crosstalk between HH- and WNT-mediated transduction pathways regulates physiological responses and is also implicated in cancer development and progression[135,140]. The activation of the canonical WNT pathway occurs through the binding of WNT ligands (WNTs) to the G protein-coupled Frizzled (FZD) receptors, which in turn bind to the low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 (REF. 141). In the presence of WNTs, β-catenin translocates into the nucleus and transactivates TCF/LEF transcription factors, thereby regulating the expression of genes involved in cell differentiation and proliferation[142].

Table 2 | **Selected G protein-coupled receptor agonists/antagonists in clinical trials***

Inhibitor	Cancer	Clinical trials
Endothelin A subtype receptor		
Zibotentan (ZD4054)	Ovarian cancer	Phase II
	Lung cancer	Phase II
	Prostate cancer	Phase I, II, III
	Advanced solid malignancies	Phase I
Atrasentan (ABT-627)	Prostate cancer	Phase II, III
	Ovarian cancer	Phase II
	Kidney cancer	Phase II
	Brain and central nervous system tumours	Phase I
Endothelin A/B subtype receptor		
Bosentan	Melanoma	Phase II
CXCR4		
BKT140	Multiple myeloma	Phase I, II
CXCR2		
CXCR2 ligands	Pancreatic cancer	Observational study
Smoothened		
IPI-926	Advanced and/or metastatic solid tumours	Phase I
BMS-833923 (XL139)	Advanced and/or metastatic solid tumours	Phase I
	Small cell lung carcinoma	Phase I
	Stomach and oesophageal neoplasms	Phase I
	Small cell lung carcinoma	Phase I
	Stomach and oesophageal neoplasms	Phase I
	Multiple myeloma	Phase I
	Leukaemia	Phase I, II
LEQ506	Advanced solid tumours	Phase I
LDE225	Advanced solid tumours	Phase I
	Basal cell carcinoma	Phase I, II
	Medulloblastoma	Phase I
	Sporadic superficial skin basal cell carcinomas	Phase II
GDC-0449 (Vismodegib)	Breast cancer	Phase I
	Pancreatic cancer	Phase I, II
	Solid tumours	Phase I
	Gastric cancer	Phase II
	Basal cell carcinoma	Phase II
	Metastatic colorectal cancer	Phase II
	Ovarian cancer	Phase II
	Lung cancer	Phase II
	Brain and central nervous system tumours	Phase II

*Information was obtained from http://www.clinicaltrials.gov.

Herpes viruses
A large family of DNA viruses that can infect and cause illness in humans.

Kaposi's sarcoma
Cancer characterized by numerous bluish-red nodules on the skin, the development of which is mainly caused by human herpes virus 8 (also known as Kaposi's sarcoma-associated herpes virus).

Burkitt's lymphoma
A non-Hodgkin's lymphoma (also called B cell lymphoma) that is associated with the Epstein–Barr virus.

Hodgkin's disease
A type of lymphoma that originates in lymph nodes and spreads to the spleen, liver and bone marrow.

β2-microglobulin
A component of major histocompatibility complex class I molecules that acts as an oncogenic factor capable of stimulating the growth and invasion of various types of tumour.

However, in the absence of WNTs, the glycogen synthase kinase 3β–axin–adenomatous polyposis coli (GSK3β–axin–APC) protein complex leads to the phosphorylation of β-catenin, which undergoes degradation by ubiquitin-dependent proteolysis[143,144]. This β-catenin-independent WNT pathway is involved in cell polarity, migration and metastasis[145] so has a functional role in tumour progression.

Numerous WNTs are highly expressed in neoplastic tissues and contribute to malignant transformation[146]. Moreover, several types of tumour release WNTs that act as stimulatory factors in a paracrine and/or autocrine manner[147]. As is the case for WNTs, several G protein-coupled FZD receptors are also highly expressed in various malignancies[148] and involved through both canonical and non-canonical WNT

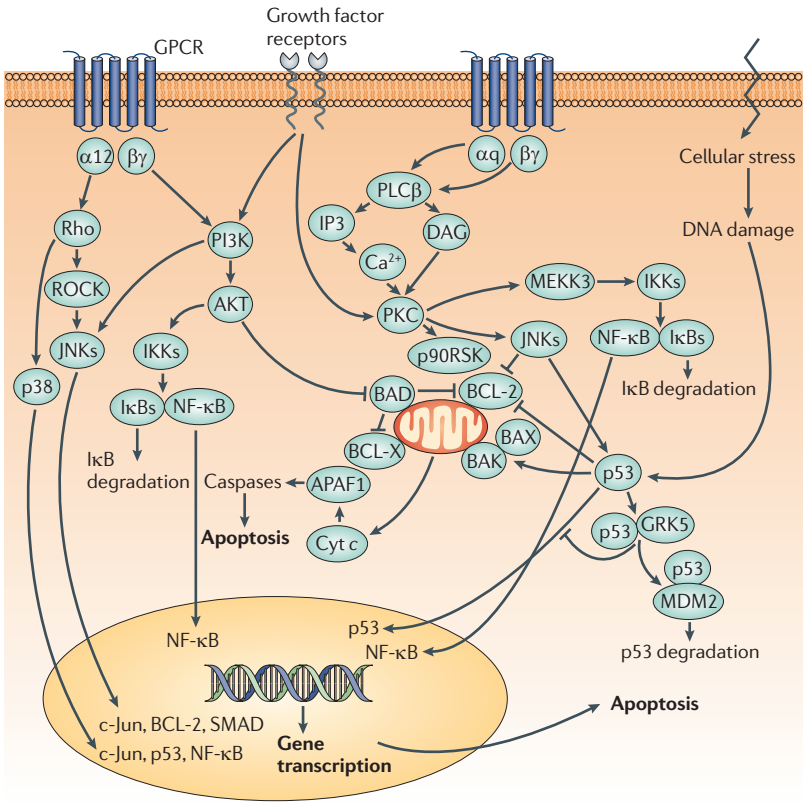


Figure 3 | **GPCRs and apoptotic pathways.** Gα12/13, Gαq and Gβγ subunits control the activity of molecules that are involved in the apoptotic process, such as small Rho GTP-binding proteins, JNK and p38 MAPKs. Gβγ subunits interact with growth factor receptors, thereby activating the PI3K–AKT survival pathway that is implicated in NF-κB-dependent transcription. Gβγ and Gαq-coupled receptors activate PLC, triggering the PKC-mediated signalling, which can also modulate the activity of NF-κB. Gα12/13, Gαq and Gβγ proteins, acting through RHO, PLC and PI3K, respectively, stimulate the JNK-dependent pathway. The activated JNKs translocate into the nucleus and phosphorylate transcription factors that are involved in the regulation of apoptosis, such as c-Jun, SMAD, BCL-2 and p53. JNKs also phosphorylate BCL-2 directly and cooperate with BCL-2 in mediating cell survival. Stress-induced apoptosis can occur through altered mitochondrial permeability, cytochrome *c* release and subsequent activation of caspases. The release of cytochrome *c* is regulated by BCL-2 family proteins, which comprise anti-apoptotic (pro-survival) as well as pro-apoptotic members. BCL-2, BCL-X and other anti-apoptotic BCL-2 family components reside in the outer mitochondrial membrane and can prevent cytochrome *c* release. The pro-apoptotic proteins BAX and BAK induce apoptosis either by directly forming pores in the mitochondria or by binding to BCL-2 and BCL-X, thereby antagonizing their anti-apoptotic activity. The BCL-2 related protein BAD promotes apoptosis by forming heterodimers with the survival proteins BCL-2 and BCL-X, preventing their binding to BAX. BAD is located on the outer mitochondrial membrane and translocates into the cytoplasm once it is phosphorylated by growth stimuli. The phosphorylation status of BAD is a key checkpoint for cell death or cell survival. Some key BCL-2 family genes are targets of p53 activity. Post-translational modifications of p53, particularly phosphorylation and acetylation, regulate its stabilization and activation. Acetylation, which is induced by many genotoxic agents, might regulate the stability of p53 by inhibiting its ubiquitylation by MDM2. GRK5 phosphorylates p53, which leads to enhanced binding to MDM2, ubiquitylation and degradation, thereby inhibiting p53-mediated apoptosis.The exact pathways that are activated are dependent on cell type. APAF1, apoptotic peptidase activating factor 1; BAD, BCL-X/BCL-2-antagonist causing cell death; BAK, BCL-2 antagonist killer 1; BAX, BCL-2-associated X protein; BCL-2, B cell CLL/lymphoma-2; BCL-X, BCL-2-related protein long isoform; Cyt *c*, cytochrome *c*; DAG, diacylglycerol; GPCR, G protein-coupled receptor; GRK5, GPCR kinase 5; IP3, inositol triphosphate; IKKs, IκB kinases; JNKs, c-JUN N-terminal kinases; MEKK3, mitogen-activated protein kinase kinase kinase 3; NF-κB, nuclear factor-κB; p90RSK, ribosomal protein S6 kinase; PI3K, phophoinositide 3-kinase; PKC, protein kinase C; PLCβ, phospholipase Cβ; Rho, RAS homology; ROCK, Rho-associated coiled-coil containing protein kinase 1.

pathways in the growth, survival, invasion and metastatic capabilities of different tumours[149–151]. In particular, the silencing of the WNT/FZD co-receptor LRP6 was associated with the repression of the WNT pathway as well as with cell proliferation in breast cancer[152], which is characterized by an aberrant activation of WNT signalling in the absence of mutations of WNT members[153].

On the basis of the important role of the WNT transduction pathway in tumour cells, several studies have attempted to discover inhibitors that are able to exert anticancer effects[154]. For example, the flavonoid quercetin[123,155], the diuretic agent ethacrynic acid[156] and the carbazole alkaloid murrayafoline A[157] elicited antitumour properties through the inhibition of the WNT–β-catenin cascade. Therefore, targeted inhibition of WNT signalling is a rational and promising approach for the therapy of various cancers, as demonstrated by the new WNT inhibitors thiazolidinediones, which decreased FZD1 and LRP6 expression as well as DNA synthesis in breast cancer cells[158,159]. Taking into account the increasing evidence implicating HH- and WNT-associated GPCRs in tumour development and progression, it is reasonable to expect that further *in vivo* studies will guarantee new therapeutic strategies based on inhibitors of HH and WNT pathways.

GPCRs and the apoptotic response

Many ligand-activated GPCRs are involved in the regulation of apoptosis in cancer cells[160–162] (FIG. 3). For instance, LPA and S1P receptors mediate either anti-apoptotic[85,163] or pro-apoptotic effects[164,165] and the receptors for orexin trigger dramatic apoptotic responses[166]. Signalling components acting downstream of the receptor or G protein — such as GPCR-bound arrestin with adaptor protein 2 — might mediate anti-apoptotic events following stimulation of multiple GPCRs, including P2Y purinergic receptors and CXCR2 (REF. 167).

GPCR connectivity to p53-mediated apoptosis. The transcription factor p53 mediates the apoptotic effects induced by diverse stress agents, such as commonly used DNA-damaging chemotherapeutics[168]. Approximately 50% of tumours have inactivating mutations of p53; however, alterations in p53-associated pathways are often detected in tumour cells without p53 mutations[169]. Several GPCRs are implicated in the apoptotic responses in cancer cells through p53-dependent transduction pathways (FIG. 3). For example, GPCR87 (also known as GPR87) has a crucial role in the p53-dependent survival of cancer cells exposed to DNA damage[170]. Moreover, LPA-dependent signalling decreases the nuclear localization and cellular abundance of p53, thereby protecting lung carcinoma cells from apoptosis[171].

p53 acts as a substrate of the GPCR kinases (GRKs) (BOX 3). For example, GRK5-mediated degradation of p53 prevents apoptosis in osteosarcoma cells and, accordingly, GRK5-deficient mice show an increase in p53 expression associated with irradiation-induced apoptosis[172]. p53 also regulates the CXCL12–CXCR4 axis. For example, in breast cancer cells, wild-type p53 lowers CXCR4 expression but mutant p53 does not[13]. In addition, the inhibitory effects on CXCL12 production

Box 3 | **G protein-coupled receptor kinases and arrestins**

G protein-coupled receptor kinases (GRKs) belong to a family of seven serine/threonine protein kinases that can phosphorylate agonist-bound G protein-coupled receptors (GPCRs), which thereby terminate their functional coupling to heterotrimeric G proteins. In particular, phosphorylated GPCRs interact with β-arrestins, then subsequently undergo desensitization[215]. However, GPCR-mediated signalling does not end with desensitization. For instance, arrestins can bind to a host of catalytically active proteins that engage with the receptor–arrestin complex. After the binding of arrestins to catalytically active proteins, other events — such as gene transcription and protein translation — are involved in the regulation of desensitization. Consequently, it is difficult to dissociate the effects of arrestin-dependent desensitization from those that might be triggered by arrestin-mediated signals. Nevertheless, studies using arrestin knockouts, G protein-uncoupled receptor mutants and arrestin pathway agonists have shown that arrestin signalling plays an important part in diverse physiological functions as well as in cancer development[216]. Changes in the desensitization process consequent to both altered GRK expression and GRK-mediated regulation of GPCRs contribute to cancer progression[172,217]. Many efforts are underway to identify small molecules that can modulate GRK activity towards the identification of alternative pharmacological routes in cancer[218].

elicited by p53 in cultured fibroblasts are further exacerbated by the p53 activating agent nutlin 3 (REF. 173), whereas in osteosarcoma SJSA1 cells and precursor-B acute lymphoblastic leukaemia/lymphoma G2 cells the invasiveness that was stimulated using medium conditioned by p53-deficient fibroblasts is reversed in the presence of CXCR4 antagonist peptides[173].

GPCR contribution to NF-κB-regulated apoptosis. A wide variety of Gαq- and Gαs-coupled GPCRs, including LPA, ET1 and angiotensin II receptors, activate the transcription factor nuclear factor-κB (NF-κB)[174] (FIG. 3), which is largely involved in inflammation and cancer[174,175]. In particular, NF-κB mediates either anti-apoptotic responses[176] or pro-apoptotic effects, depending on the stimulus and the cell context[177]. Numerous evidence associated NF-κB activity with adaptor and scaffold proteins, including β-arrestin 2, BCL-10, MALT1 and CARMA3, which act as essential signalling transducers of GPCRs[178–180]. There are several studies suggesting that other GPCRs and their ligands can activate NF-κB in cancer cells, including angiotensin II/AT1R-mediated and NF-κB-dependent proliferation of gastric cancer cells[181], bradykinin/B2 receptor-mediated migration of chondrosarcoma cells[182] and adenosine/A3 receptor-mediated repression of colon carcinoma cells[183]. Similar findings were evidenced *in vivo* for adenosine receptors. For example, adenosine Gαs- and Gαi-coupled GPCRs regulate NF-κB in the development of diverse malignancies[174] and the adenosine A3 receptor agonist IB-MECA decreased NF-κB expression in colon carcinoma xenografts[184]. Altogether, the regulatory role played by GPCRs on NF-κB activity might be a valuable target towards drug discovery and/or the use of drugs in combination for innovative treatments in cancer patients.

GPCR action in the tumorigenesis of viral factors

Herpes viruses contain genes that encode constitutively active GPCRs, and these contribute to viral replicative success and pathogenesis, as well as virally induced oncogenesis[185]. For example, the aetiological agent for Kaposi's sarcoma is the Kaposi's sarcoma-associated herpes virus (KSHV; also known as human herpes virus 8 (HHV8)), which encodes over 80 viral polypeptides, including the viral GPCR ORF74 (also known as vGPCR) that is involved in the pathogenesis of Kaposi's sarcoma[186–188]. vGPCR is a constitutively active homologue of the human IL-8 receptor CXCR2 that has promiscuous chemokine-binding activity[189]. Although ligand binding is not required for vGPCR-mediated signalling, the cognate chemokines modulate vGPCR activity *in vitro* and *in vivo*[189,190]. vGPCR stimulates cell proliferation, angiogenesis[191] and mediates cell transforming activity and anti-apoptotic effects exerted by KSHV[192,193].

Transgenic mice expressing vGPCR developed Kaposi's sarcoma-like lesions in multiple organs[193,194] and the injection of vGPCR-expressing mouse fibroblasts into the flank of nude mice caused vascularized tumours[192]. The 1α,25-dihydroxyvitamin D(3) and its analogue TX527 elicited both *in vitro* and *in vivo* repressive effects on the proliferation of vGPCR-transformed endothelial cells[195], providing experimental evidence for the discovery of novel therapies aimed at targeting vGPCR signalling[188]. Remarkably, vGPCR can stimulate the HIF1α-dependent transcription of VEGF, which occurs through a hypoxia response element located within the VEGF promoter sequence[196]. These findings may connect different players leading to cancer progression, such as GPCRs involved in the viral tumorigenesis, hypoxia-associated factors and mediators of angiogenic processes.

A further herpes virus, the Epstein–Barr virus (EBV), exhibits peculiar growth-transforming activity in Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma[197]. In particular, the EBV-encoded open reading frame BILF1 encodes a functional Gαi-coupled receptor, which has a relevant role in EBV infection and associated malignancies[198]. For example, this viral GPCR stimulates NF-κB signalling and inhibits RNA-dependent protein kinase activation in tumour cells[199]. BILF1 is the only human herpes virus-encoded GPCR that can contribute to immune evasion by targeting major histocompatibility complex class I molecules[200]. Similar to BILF1, the activation of CXCR4 by its cognate ligand CXCL12 can induce the downregulation of cell surface HLA-I expression[201]. Mechanistically, this process involves the physical association of CXCR4 with the β2-microglobulin, ubiquitylation of the HLA-I heavy chain and endocytosis followed by degradation. BILF1-induced degradation of HLA-I mimics this process as BILF1 binds to the HLA-I–β2-microglobulin complex, accelerates loss of HLA-I molecules from the cell surface and markedly reduces the half-life of HLA-I molecules[201].

The human cytomegalovirus (HCMV), a herpes virus detected in malignancies such as glioblastoma[202], colon[203] and breast cancer[204], infects tumour cells and enhances cell proliferation, angiogenesis and resistance to apoptosis[205,206]. Among the four HCMV-encoded GPCRs, the chemokine receptor US28 activates proliferative signalling pathways, thereby stimulating tumorigenesis[207]. In particular, US28 activates the Gαq-linked pathway and promotes DNA synthesis, cyclin D1 upregulation and VEGF secretion, which lead to tumour formation *in vitro* and *in vivo*[207]. US28 also modulates

the expression of diverse tumorigenic proteins such as cyclooxygenase 2, which is a key mediator of inflammatory diseases and a major determinant in many tumours[208]. Accordingly, the inhibition of cyclooxygenase 2 was associated with a marked delay in the onset of tumour formation in nude mice that were injected with US28-transfected NIH-3T3 cells[208]. In addition, US28 modulates the immune responses by transcriptionally regulating the expression of MCP1 (monocyte chemoattractant protein 1) as well as binding to and internalizing MCP1 and RANTES (regulated on activation, normal T cell expressed and secreted)[201].

On the basis of these data, viral GPCRs are currently considered promising drug targets for innovative treatments in infectious diseases and related malignancies[185]. GPCRs that are encoded by herpes viruses are exciting examples of the pathophysiological relevance of constitutive GPCR activity. Current efforts directed towards the identification of drugs acting selectively as antagonists of viral GPCRs are promising for new drug discoveries and therapeutic interventions in patients with cancer.

Concluding remarks and future perspectives

GPCRs are key players in the regulation of various pathophysiological responses, including cancer development and progression. Currently, the pharmacological manipulation of diverse GPCRs is an excellent option to block tumorigenic signals, making GPCR-mediated functions promising therapeutic targets in drug development towards innovative intervention in cancer. Moreover, the complex crosstalk between GPCR signalling and diverse transduction pathways, such as growth factor receptors[20,24], which leads to cancer progression, may provide a golden opportunity for targeting interacting molecules with selected inhibitors.

For example, recent evidence[57] that GPER — a GPCR that can mediate oestrogen action — can act as a transcription factor directly interacting with EGFR has extended our current knowledge regarding the mode of action of GPCRs, suggesting unexpected possibilities for therapeutic intervention. Supporting these challenges, the ET_AR antagonists ZD4054 and atrasentan used in combination with the EGFR inhibitor gefitinib and the monoclonal HER2-specific antibody trastuzumab, respectively, show a strong ability to repress the proliferation and invasion of cancer cells[35,40]. ZD4054 and atrasentan also show good antitumour efficacy in clinical trials; however, further clinical studies are required to evaluate whether each ET_AR antagonist used in combination with EGFR inhibitors may be more effective in cancer patients than as a single treatment.

An ideal further approach to assess new therapeutic strategies could combine the manipulation of ligand–GPCR interactions with the inhibition of ligand biosynthesis. In this regard, it is noteworthy that the high secretion of LPA by cancer cells is coupled with an aberrant expression of GPCRs that mediate LPA signalling, highlighting the need for new therapeutics that target both biological hallmarks of LPA-stimulated cancer progression[108]. Although the development of chemicals that act as selective inhibitors of GPCRs has been challenging, various high-throughput screening technologies have strengthened current drug-discovery efforts[209,210]. Additionally, the high number of orphan GPCRs is an attractive area of research, as demonstrated in recent years by the identification of many ligands for previously orphaned GPCRs.

We can expect that the improved understanding of the molecular pharmacology of GPCRs, coupled with a plethora of novel high-throughput screening technologies, will soon lead to the discovery of an entirely new generation of GPCR-based therapeutics, including the current orphan GPCRs, which will drive major clinical benefits for cancer patients. Huge efforts will be required to selectively target GPCRs with specialized functions. Consequently, leaving unaffected individual physiological activities, the risk of side effects will be minimized. The discrete manipulation of the GPCR signalling network — defined in a new way as a GPCR interactome — will thereby provide rational basis for the design of more focused clinical trials towards the introduction of cancer-tailored treatments.

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The authors declare no competing financial interests.

CASE HISTORY

The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor

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Abstract | The activated serine protease factor Xa is a promising target for new anticoagulants. After studies on naturally occurring factor Xa inhibitors indicated that such agents could be effective and safe, research focused on small-molecule direct inhibitors of factor Xa that might address the major clinical need for improved oral anticoagulants. In 2008, rivaroxaban (Xarelto; Bayer HealthCare) became the first such compound to be approved for clinical use. This article presents the history of rivaroxaban's development, from the structure–activity relationship studies that led to its discovery to the preclinical and clinical studies, and also provides a brief overview of other oral anticoagulants in advanced clinical development.

Thromboembolic disorders
A group of conditions characterized by an increased incidence of thrombi in the vasculature, such as deep-vein thrombosis, pulmonary embolism, systemic embolism or coronary and cerebral ischaemia.

Unfractionated heparin
(UFH). An anticoagulant administered intravenously or subcutaneously. It binds to antithrombin, greatly increasing its activity and resulting in the inhibition of factors Xa, IXa, XIa, XIIa and thrombin (factor IIa).

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Anticoagulants are used for the prevention and treatment of venous and arterial thromboembolic disorders. Many approaches have been explored in the development of antithrombotic drugs that inhibit enzymes in the coagulation pathways. However, most currently approved drugs for the prevention and treatment of thromboembolic disorders have been on the market for a long time, in some cases for decades. Unfractionated heparin (UFH), which was discovered in 1916 (REF. 1) targets multiple factors in the coagulation cascade[2], but has a number of limitations, including a parenteral route of administration, frequent laboratory monitoring of coagulation activity and the risk for patients of developing potentially life-threatening heparin-induced thrombocytopaenia[2,3]. Low-molecular-weight heparins (LMWHs), which were developed in the 1980s, promote the inactivation of both thrombin (factor IIa) and, to a greater extent, factor Xa[2].

LMWHs have largely replaced UFH owing to their lower risk of causing bleeding, lower levels of binding to plasma proteins and endothelium, good bioavailability, longer half-life and superior pharmacokinetic properties compared with UFH[2]. However, their use remains limited because of the need for parenteral administration[2], which can be inconvenient, especially in an outpatient setting, with patients needing to be trained to self-inject after discharge, and nurse visits required for those unable to do so[4]. Both UFH and LMWHs are indirect inhibitors of coagulation, and their activity is mediated by plasma cofactors, principally antithrombin and, to a lesser extent for UFH, heparin cofactor II (REF. 2).

Warfarin, the prototype vitamin K antagonist (VKA), was originally discovered in 1941 (REF. 5). Until recently, the VKAs were the only available oral anticoagulants, as well as the most frequently prescribed. However, VKAs have numerous well-documented drawbacks, including unpredictable pharmacokinetics and pharmacodynamics, a slow onset and offset of action, a narrow therapeutic window, multiple food–drug and drug–drug interactions[6], and considerable inter-individual and intra-individual variability in dose response. In addition, regular coagulation monitoring and dose adjustment are required to keep patients within the target international normalized ratio (INR) range, usually 2.0–3.0, which can be costly[6]. Furthermore, establishing the optimal dose of warfarin is complicated by variations in warfarin sensitivity due to common genetic polymorphisms, particularly in *CYP2C9* and *VKORC1* (REF. 7). Attempts have been made to use pharmacogenetics to estimate dose, although the clinical value of such assessments is debatable[8]. Nonetheless, LMWHs and VKAs are still the basis of contemporary thromboprophylaxis and treatment. However, the difficulties and shortcomings that surround the practicalities and clinical management of these established anticoagulants — particularly parenteral administration, the need for monitoring and the lack of predictable response — has spurred the development of new agents that are less burdensome for the patient and health-care system, and address both patients' and physicians' unmet needs. TABLE 1 contrasts the characteristics of the VKAs with those of a

Table 1 | **Comparison of an ideal anticoagulant and a vitamin K antagonist**

Property	Ideal anticoagulant	Vitamin K antagonist
Administration	Oral	Oral
Onset/offset of action	Rapid (several hours)	Slow (several days)
Therapeutic window	Wide	Narrow
Variability in dose response	Little or no inter-individual or intra-individual variability	Considerable inter-individual and intra-individual variability
Interactions	Little or no interaction with food or other drugs	Multiple interactions with food and other drugs
PK/PD	Predictable	Unpredictable and variable
Coagulation monitoring	No routine monitoring required	Regular monitoring required
Dose adjustment	None required	Required
Efficacy	Highly effective in reducing thromboembolic events	Effective in reducing thromboembolic events when properly controlled
Safety profile	Good, especially with regard to bleeding	Difficulties in maintaining patients within the target therapeutic range (INR 2.0–3.0); contributes to an increased risk of bleeding

INR, international normalized ratio; PK/PD, pharmacokinetics/pharmacodynamics.

hypothetical 'ideal' anticoagulant, and FIG. 1 chronicles the historical development of anticoagulants.

Despite the accumulated knowledge on the coagulation system (FIG. 2), its complexity has presented numerous obstacles to the discovery and development of potent anticoagulants that are both effective and safe. In recent years, research has focused on new classes of anticoagulants that target a specific coagulation enzyme or step in the coagulation cascade[9,10], including inhibitors of the factor VIIa–tissue factor complex[10], inhibitors of factor IXa[11,12] and factor XIa[13,14], direct thrombin inhibitors[15,16], synthetic indirect and direct inhibitors of factor Xa (activated factor X)[17–20], and recombinant soluble thrombomodulin[21,22]. In addition, recombinant activated protein C (APC) mitigates the procoagulant state associated with sepsis[23,24].

In the search for new anticoagulant drugs, the activated serine protease factor Xa is a particularly promising target and has attracted great interest in recent years[18,25–27]. This article describes the discovery and development of the first oral, direct factor Xa inhibitor to be approved for clinical use — rivaroxaban (Xarelto; Bayer HealthCare). Rivaroxaban was approved by the European authorities in 2008 for the prevention of venous thromboembolism (VTE; comprising deep-vein thrombosis (DVT) and pulmonary embolism) after elective hip or knee replacement. The article then briefly considers the future clinical potential of rivaroxaban and other factor Xa inhibitors currently in advanced clinical development.

Factor Xa: function and biology

Factor X has long been known to have a key role in haemostasis[28] and factor Xa plays a central part in the blood coagulation pathway by catalysing the production of thrombin, which leads to clot formation and wound closure[20] (FIG. 2). Conversely, deficiency of factor Xa may disturb haemostasis. In the very rare factor X deficiency disorder (for which 1 in 500,000 is homozygous and 1 in 500 heterozygous), very low plasma and activity levels of factor Xa manifest as severe bleeding tendencies[29–31]. Studies of variants of factor X deficiency indicate that factor X plasma activity levels must be as low as 6–10% of the normal range (approximately 50–150% of the population average) to be considered a mild deficiency; cases with factor X activity levels below 1% are considered to be severe[29,32]. Thus it seems that factor X activity can be markedly suppressed without affecting haemostasis. An ideal anticoagulant would prevent thrombosis without inducing systemic hypocoagulation, and would thereby avoid unintended bleeding complications. Therefore, a factor Xa inhibitor could potentially have the properties of a desirable anticoagulant.

Validating factor Xa as a drug target

The first factor Xa inhibitors. Although factor Xa was identified as a promising target for the development of new anticoagulants in the early 1980s, the viability of factor Xa inhibition was not tested before the end of that decade. In 1987, the first factor Xa inhibitor, the naturally occurring compound antistasin, was isolated from the salivary glands of the Mexican leech *Haementeria officinalis*[33,34]. Antistasin is a 119 amino-acid polypeptide; kinetic studies revealed that it is a slow, tight-binding, potent factor Xa inhibitor (inhibition constant (K_i) of 0.3–0.6 nM) that also inhibits trypsin (half maximal inhibitory concentration (IC_{50}) is 5 nM in the presence of 1 nM trypsin)[35]. Another naturally occurring factor Xa inhibitor, the tick anticoagulant peptide (TAP), a single-chain, 60 amino-acid peptide, was isolated in 1990 from extracts of the soft tick *Ornithodoros moubata*[36]. Similarly to antistasin, TAP is a slow, tight-binding inhibitor of factor Xa (K_i of ~0.6 nM).

TAP[37] and recombinant forms of antistasin[38] and TAP[38–40] were used to validate factor Xa as a viable drug target and to improve understanding of the role of factor Xa in thrombosis. The antithrombotic effects of these compounds were compared with those of direct thrombin inhibitors and of indirect thrombin and factor Xa inhibitors (that is, UFH) in animal models of thrombosis. These studies suggested that direct factor Xa inhibitors might be a more effective approach to anticoagulation[37,39], and might also offer a wider therapeutic window, particularly with regard to primary haemostasis[40,41].

In vitro *and* in vivo *studies.* Clot-bound factor Xa was shown to be enzymatically active *in vitro* and able to activate prothrombin to thrombin[42]. In addition, factor Xa was found to be an important contributor to clot-associated procoagulant activity *in vitro*[43]. Clot-bound factor Xa activity was resistant to inhibition by antithrombin[42], suggesting that the ability to directly inhibit clot-associated factor Xa, with no requirement for a cofactor, could provide an effective and highly localized approach to the prevention of thrombus growth. The clot-associated

Heparin-induced thrombocytopaenia
The process by which antibodies against the complex of heparin and platelet factor 4 activate platelets, resulting in a decrease in platelet numbers of more than 50%.

Low-molecular-weight heparins
(LMWHs). A class of anticoagulants derived from unfractionated heparin by chemical or enzymatic degradation. They induce a conformational change in antithrombin that greatly increases its anticoagulant activity.

Thrombin
Thrombin (also known as factor IIa) is the terminal enzyme of the coagulation cascade and converts fibrinogen into fibrin, which forms clot fibres. Thrombin also activates several other coagulation factors, as well as protein C.

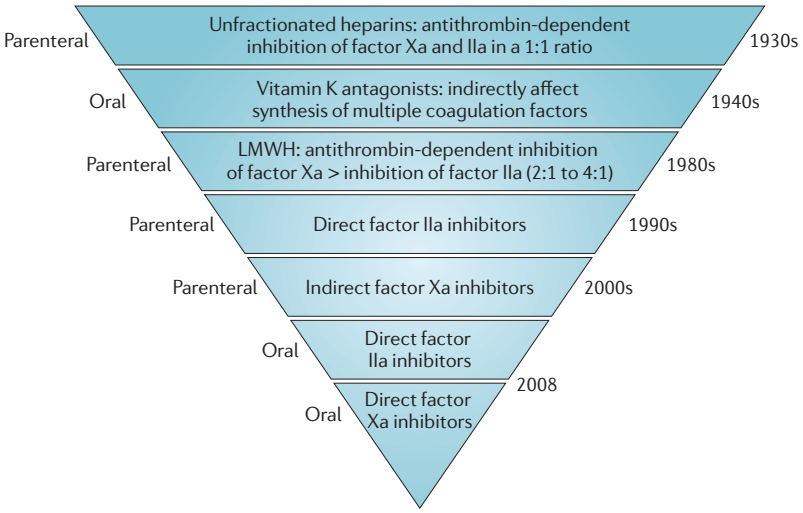


Figure 1 | **Development of anticoagulants over the past century.** The timeframe starts with the discovery of the heparins and the vitamin K antagonists, followed by the low-molecular-weight heparins. These were followed, in turn, by the discovery of the parenteral direct thrombin inhibitors, the development of the indirect factor Xa inhibitor, fondaparinux, and the work of the current decade that has resulted in oral direct inhibitors of both thrombin (factor IIa) and factor Xa. The inverted triangle reflects the narrowing of action and increasing specificity of the anticoagulants. LMWH, low-molecular-weight heparin.

generation of fibrinopeptide A (a byproduct of fibrinogen cleavage by thrombin) was inhibited to the same extent by both recombinant TAP and hirudin, a direct thrombin inhibitor. This observation suggested that procoagulant activity in the clot was due to *de novo* activation of prothrombin to thrombin, rather than to the activity of pre-existing thrombin[43]. It was subsequently shown that inhibition of factor Xa by recombinant TAP provided sustained *in vitro*[44] and *in vivo*[45] inhibition of clot-associated procoagulant activity, which may, in turn, protect against ongoing coagulation after cessation of anticoagulant treatment.

Overall, these data suggested that direct factor Xa inhibitors might not be linked to the phenomenon of 'rebound' thrombosis that was associated with the direct and indirect thrombin inhibitors previously under investigation[46,47]. Rebound thrombosis can be thought of as a transient increase in thromboembolic events occurring shortly after the withdrawal of an antithrombotic medication[46,48]. Furthermore, low concentrations of a direct thrombin inhibitor may partly suppress the negative feedback on coagulation by APC, in contrast to factor Xa inhibition, which does not seem to measurably affect the thrombin–thrombomodulin–APC system[49]. However, whether these experimental observations have any clinical relevance remains to be determined.

The first synthetic factor Xa inhibitors

Although antistasin and TAP provided support for the concept of factor Xa inhibition, development of these compounds was discontinued. The reasons were never disclosed. Nonetheless, the encouraging results from studies using recombinant versions of the natural factor Xa inhibitors prompted several pharmaceutical companies to initiate chemistry programmes to develop selective, small-molecule, direct inhibitors of factor Xa, such as DX-9065a[50] and YM-60828 (REFS 51,52) (FIG. 3). Both these compounds contain a highly basic amidine residue, designed as mimics for the arginine of the natural substrate prothrombin.

DX-9065a, a widely studied non-peptidic small molecule, shows rapid, direct and reversible binding kinetics for factor Xa (K_i of 41 nM)[53]. At physiological pH it is a zwitterion with high water solubility and low lipophilicity[54]. However, human oral bioavailability was only 2–3%[54]. A small Phase II study was conducted in patients with non-ST-elevation acute coronary syndrome (ACS) who were randomized to low- or high-dose intravenous DX-9065a or UFH[55]. A non-significant trend was observed towards a reduction in ischaemic events and bleeding for DX-9065a compared with UFH[55].

Because of the success of indirect dual factor Xa and thrombin inhibitors, such as LMWHs, indirect inhibitors of factor Xa with greater selectivity, such as fondaparinux (Arixtra; GlaxoSmithKline)[56,57], were developed in parallel with direct factor Xa inhibitors.

Both UFH and LMWHs contain a unique pentasaccharide sequence that mediates binding to antithrombin. Binding induces a conformational change in antithrombin that potentiates its ability to inhibit coagulation factors. Inhibition of thrombin occurs through heparin chains of sufficient length to bridge antithrombin to thrombin in a ternary complex, after which antithrombin binds covalently to thrombin and the heparin chain dissociates[2]. However, such bridging is not necessary for antithrombin to be able to inhibit factor Xa. Most LMWH chains are too short to catalyse thrombin inhibition, but can nonetheless promote the inhibition of factor Xa. Thus, UFH has an anti-Xa to anti-IIa ratio of 1:1, LMWHs have anti-Xa to anti-IIa ratios from 2:1 to 4:1, depending on the molecular weight distribution of the preparation[2].

Fondaparinux is an analogue of the pentasaccharide sequence required to promote the binding of antithrombin to factor Xa[2]. The pentasaccharide structure is too short to enable bridging between antithrombin and thrombin. As a result, fondaparinux exclusively potentiates the anti-factor Xa activity of antithrombin and has no effect on thrombin[2]. The efficacy and safety of fondaparinux for the prevention of VTE after major orthopaedic surgery were investigated in four randomized, Phase III trials in patients undergoing surgery for hip fracture[58], hip replacement[59,60] and knee replacement[61]. A meta-analysis of these trials showed the superior efficacy of fondaparinux over the LMWH enoxaparin (Lovenox/Clexane; Sanofi–Aventis) in reducing the incidence of VTE. However, major bleeding occurred more frequently in the fondaparinux-treated group (P=0.008), although the incidence of clinically relevant bleeding (bleeding leading to death, reoperation or in a critical organ) did not differ between the treatment groups[62]. Fondaparinux provided the proof of principle that selective inhibition of factor Xa could provide clinically effective anticoagulation.

Antithrombin
An endogenous glycoprotein that binds covalently to thrombin and other coagulation factors, resulting in their inhibition. Antithrombin functions as a natural anticoagulant, and its inhibitory action is accelerated by heparin.

Warfarin
A vitamin K antagonist that is currently the most commonly used oral anticoagulant.

Vitamin K antagonist
A class of compounds that inhibit the vitamin K-dependent carboxylation of specific coagulation factors, resulting in decreased levels of the affected coagulation factors, leading to anticoagulation.

Therapeutic window
The interval between the lowest dose of a drug that is sufficient for clinical effectiveness and a higher dose at which adverse events or toxicity become unacceptable.

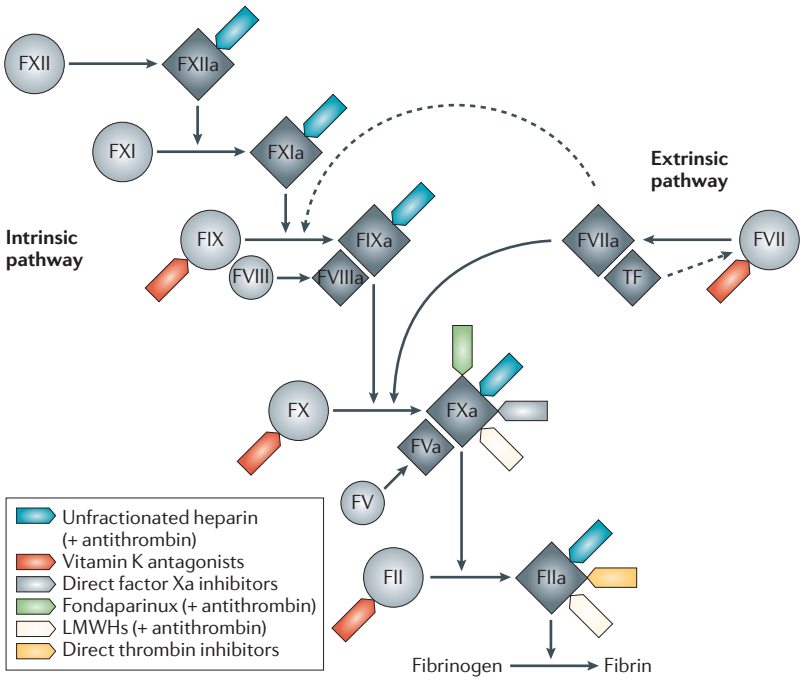


Figure 2 | **Simplified schematic for the blood coagulation cascade.** The figure identifies the target points of various anticoagulants and illustrates that factor X (FX) can be activated through either the intrinsic or the extrinsic pathway. The factor VIIa–tissue factor (TF) complex (extrinsic tenase) activates both factor IX and factor X, as well as factor VII itself (dashed arrow)[150,151]. Initiation of either pathway activates the inactive precursor, factor X, to factor Xa. This makes factor Xa a desirable intervention point for novel anticoagulants, because it occupies a central role in the blood-coagulation pathway[20]. Furthermore, in addition to initiation, both the intrinsic and extrinsic pathways lead to the propagation and amplification of coagulation through the activation of factor X. During the initiation phase of coagulation, the factor Xa produced generates some thrombin (factor IIa). This initial thrombin activates factor XI, and factors V and VIII, to factor XIa and the activated cofactors, factor Va and VIIIa, respectively. It also activates platelets (not shown), which are required for the formation of the intrinsic tenase (factor VIIIa–factor IXa) and the prothrombinase (factor Va–factor Xa) complexes. The prothrombinase complex, on the platelet surface, is substantially more efficient than free factor Xa at activating prothrombin to thrombin; the rate of thrombin formation is increased by approximately 300,000-fold over the rate with factor Xa alone[152]. Thrombin is the principal enzyme involved in the formation, growth and stabilization of thrombi. Thrombin mediates the conversion of fibrinogen to fibrin, the activation of factor XIII (which crosslinks and stabilizes fibrin), the activation of platelets and the above-mentioned feedback-activation of upstream coagulation factors, factor V, factor VIII and factor XI[151,153], resulting in the amplification of its own formation[150,154]. Because one molecule of factor Xa catalyses the formation of approximately 1,000 thrombin molecules[25,154], this amplification can be substantial. Compared with thrombin, factor Xa is thought to have fewer effects outside coagulation and, together with factor Va (as the prothrombinase complex), acts mainly to convert prothrombin to thrombin. Specific inhibition of factor Xa does not affect pre-existing thrombin but does inhibit thrombin generation[155]. Conversely, although thrombin generation is delayed in the presence of a thrombin inhibitor, the amount of thrombin generated is only reduced at higher inhibitor concentrations, albeit in a concentration-dependent manner[156]. LMWH, low-molecular-weight heparin.

Numerous investigations, performed with both direct and indirect factor Xa inhibitors, showed that inhibition of factor Xa produces antithrombotic effects by decreasing the generation of thrombin, thus diminishing thrombin-mediated activation of both coagulation and platelets without affecting the activity of existing thrombin. However, the residual thrombin generated seems to be sufficient to ensure normal systemic haemostasis, possibly because thrombin has a very high affinity for platelet receptors and minimal amounts of thrombin can provide sufficient platelet activation, thus contributing to a favourable efficacy/safety ratio[20]. On the basis of these findings, in the mid-1990s, it seemed that small-molecule, direct factor Xa inhibitors could potentially provide an advantage over the antithrombotic therapies available at that time. Several new factor Xa inhibitors are now in clinical development. These compounds, which include rivaroxaban and the other direct factor Xa inhibitors discussed later, represent new chemical entities with similar binding modes at the active site of factor Xa.

The discovery of rivaroxaban

From the first screening hit to the oxazolidinone lead. When we initiated the factor Xa programme at Bayer HealthCare in 1998, no orally active factor Xa inhibitors with sufficient antithrombotic activity were known. All known potent inhibitors that had previously been investigated contained an amidine group or other highly basic residues, which were designed to act as mimics for an arginine present in the natural substrate, prothrombin. For a long time, these mimics were thought to be a prerequisite for high binding affinity[63]. However, we found that strongly basic mimics contribute to poor oral absorption, an observation also later published by others[64,65].

High-throughput screening of approximately 200,000 compounds revealed several hits that selectively inhibited the cleavage of a chromogenic substrate by human factor Xa[63]. The most potent of these hits was on a minor impurity in a combinatorial library — a phosphonium salt with an IC_{50} of 70 nM (compound **1** in FIG. 4). We proposed that this positively charged phosphonium moiety might serve as an arginine mimic and could be interchangeable with an amidine group. This resulted in the synthesis of lead compound **2** with similar potency (IC_{50} of 120 nM)[63]. Further optimization resulted in the synthesis of compounds of the isoindolinone class, among which imidazoline **3** (FIG. 4) was the most potent (IC_{50} of 2 nM).

To achieve oral bioavailability, we explored less basic or non-basic amidine replacements. Aminopyridines, such as compound **4** (IC_{50} of 8 nM; FIG. 4), showed IC_{50} values in the low nanomolar range; however, although they were less basic, oral absorption remained insufficient. The less potent pyridylpiperazine derivative **5** (IC_{50} of 48 nM), meanwhile, revealed a significantly improved oral bioavailability of 38% in rats. Although we had demonstrated that improved oral bioavailability could be achieved using less basic amidine replacements, we were not able to meet our target of identifying factor Xa inhibitors with both high potency and sufficient oral bioavailability in the isoindolinone class of compounds. However, from these studies we did learn that broad variations in the benzylamidine part were permissible, but found that there was a very steep structure–activity relationship (SAR) for the chlorothiophene carboxamide moiety, which was already present in the lead structure.

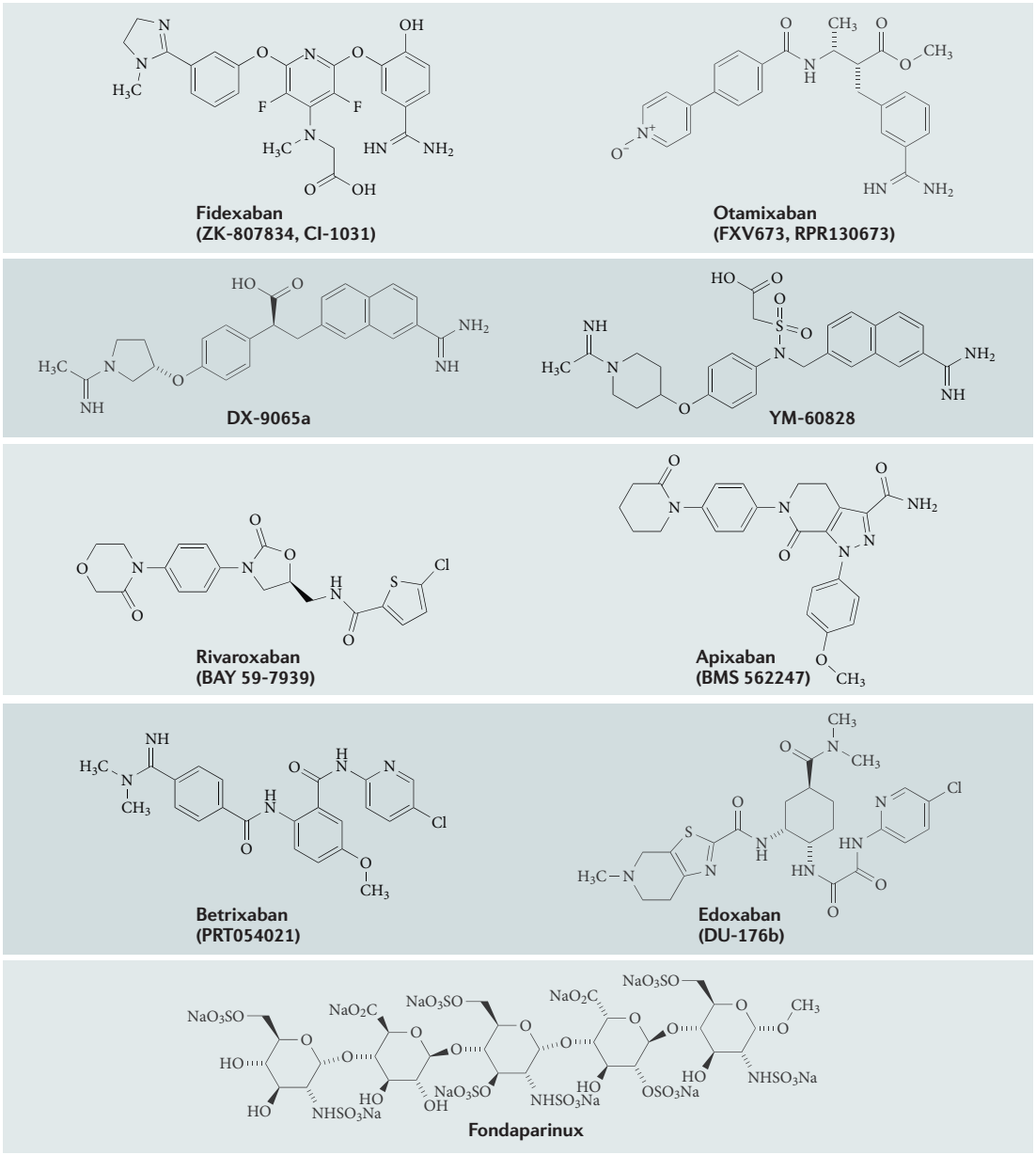


Figure 3 | **Structures of various factor Xa inhibitors.** Fidexaban, otamixaban, DX-9065a, YM-60828, rivaroxaban (Xarelto; Bayer HealthCare), apixaban (Pfizer/Bristol-Myers Squibb), betrixaban and edoxaban are shown. The structure of YM150 has not been published. Not all of these compounds are still in clinical development (for example, fidexaban and DX-9065a) but study of their structures contributed to our understanding of the structure–activity relationships and pharmacology of factor Xa inhibitors. Key features to note are the highly basic arginine mimetic amidine groups as P1 moieties in fidexaban, otamixaban, DX-9065a and YM-60828, which contribute to poor oral bioavailability. These can be contrasted with the non-basic P1 moieties: the chlorothiophene moiety in rivaroxaban, the methoxyaryl group in apixaban and the chloro-substituted pyridine rings in edoxaban and betrixaban, all of which allow improved oral bioavailability. Other synthetic factor Xa inhibitors have also been developed[26]. The structure of the synthetic indirect factor Xa inhibitor, fondaparinux (Arixtra; GlaxoSmithKline), is also shown. This is an analogue of the heparin pentasaccharide sequence required to mediate the conformational change in antithrombin and subsequent binding to factor Xa[2].

International normalized ratio
(INR). Because prothrombin time-test results vary according to the activity of the thromboplastin used, the INR conversion is used to normalize results for any thromboplastin preparation. It is valid only with vitamin K antagonists.

Thromboprophylaxis
A measure taken to prevent the development of a thrombus. It can be pharmaceutical or mechanical.

Tissue factor
A cell-membrane-bound receptor protein that is exposed to the circulating blood during vessel injury. Pre-existing factor VIIa in the blood binds to tissue factor, initiating the coagulation cascade.

Direct thrombin inhibitors
A class of anticoagulants that bind directly to thrombin and block the interaction with its substrate, fibrinogen, thereby inhibiting the generation of fibrin and clot formation.

Thrombomodulin
A membrane-bound thrombin receptor that, when bound to thrombin, functions as a cofactor in the thrombin-induced activation of protein C.

Protein C
The inactive precursor of activated protein C (APC). APC, with its cofactor protein S, inactivates factor Va and factor VIIIa, thus providing an important anticoagulant feedback function.

Factor Xa inhibitor
A class of anticoagulants that inhibit factor Xa in the coagulation cascade, either by binding directly, or indirectly through antithrombin. Inhibition of factor Xa reduces the production of thrombin.

Venous thromboembolism
(VTE). A condition in which a blood clot (thrombus) that has formed in the venous system breaks free (becoming an embolus) and migrates through the circulation to lodge in and block another blood vessel.

The failure to find a compound with sufficient potency and bioavailability could have ended the project. However, we decided instead to re-evaluate the weaker screening hits. The oxazolidinone (compound **6**; FIG. 4) was a weak factor Xa inhibitor, with an IC_{50} of 20,000 nM. Considering the importance of the chlorothiophene residue in the class of compounds studied previously, we replaced the thiophene moiety of compound **6** with a 5-chlorothiophene group, thereby creating lead compound **7** (FIG. 4). This had a >200-fold higher potency (IC_{50} of 90 nM) and did not include any basic group[63].

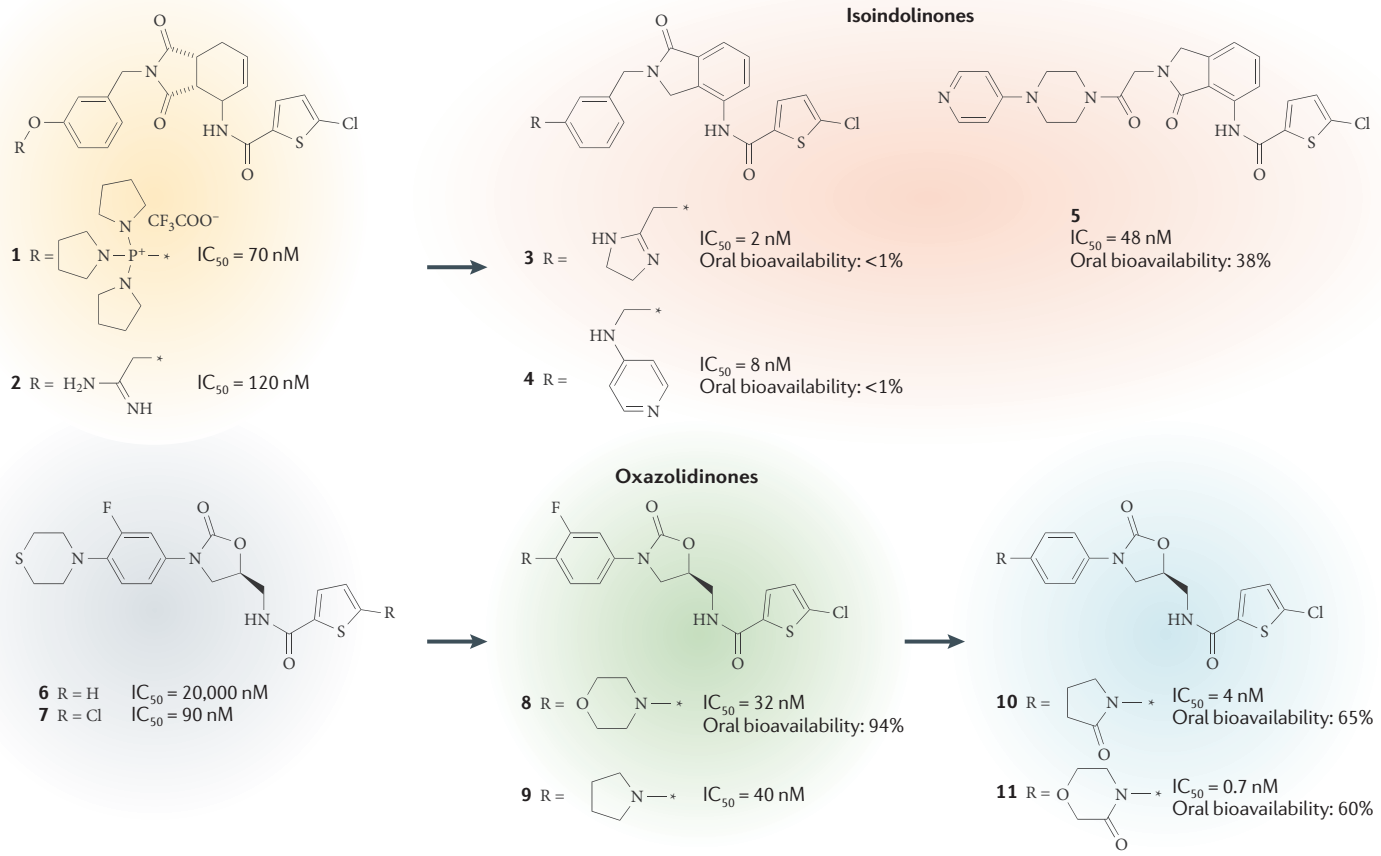


Figure 4 | **Optimization of oxazolidinone factor Xa inhibitors.** Optimization of oxazolidinone factor Xa inhibitors resulted in the discovery of rivaroxaban (compound **11**)[63]. Half-maximal inhibitory concentration (IC_{50}) values are for the inhibition of factor Xa activity; oral bioavailability is shown for rats.

On the basis of this promising lead, a medicinal chemistry programme was followed that focused on further improving the potency of the oxazolidinone class without compromising its pharmacokinetic profile.

The optimization programme leading to rivaroxaban. The starting point of these investigations was the thiomorpholine group; morpholine and pyrrolidine derivatives (compounds **8** and **9**; FIG. 4) showed some improvement in potency (IC_{50} values of 32 nM and 40 nM, respectively). Although not sufficiently potent, compound **8** was the first compound to show a favourable pharmacokinetic profile, with a high oral bioavailability of 94% in rats. An ortho-substitution led to the pyrrolidinone derivative **10**, with significantly improved potency (IC_{50} of 4 nM) and an oral bioavailability of 65%. However, the *in vivo* antithrombotic potency of this compound, evaluated in an arteriovenous shunt model in anaesthetized rats, was estimated to be too low[63]. Our knowledge of the SAR then led us to design the morpholinone residue, resulting in compound **11** (rivaroxaban; FIG. 4), for which binding to factor Xa depends on the (*S*)-configuration at the oxazolidinone core. Rivaroxaban showed increased potency *in vitro* (IC_{50} of 0.7 nM) and *in vivo* after oral administration in rats[63]. This compound also showed favourable oral bioavailability (60% in rats and 60–86% in dogs)[63].

The optimization programme was continued in order to gain a more in-depth understanding of the SAR. However, further variations evaluated at this time, such as substitution of the aryl ring or less lipophilic replacements for the chlorothiophene carboxamide moiety, did not result in further improvements[63], and the morpholinone derivative **11** (rivaroxaban) remained the most attractive candidate. The X-ray crystal structure of rivaroxaban in complex with human factor Xa[63] (BOX 1) helped us to understand its binding mode and to explain the steep SAR observed earlier. A similar binding mode has been reported for several other factor Xa inhibitors[66–70] (BOX 1).

With its combination of high binding affinity and good oral bioavailability, rivaroxaban was identified as the drug candidate for further development.

Preclinical studies

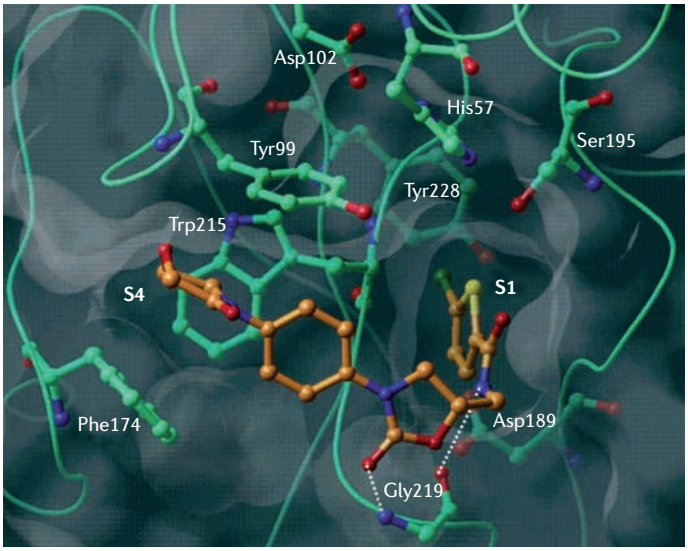
Rivaroxaban is a direct, specific factor Xa inhibitor that, unlike indirect agents such as fondaparinux, does not require a cofactor[71]. *In vitro* kinetic studies showed that the inhibition of human factor Xa by rivaroxaban was competitive (K_i of 0.4 nM), with >10,000-fold greater selectivity for factor Xa than for other serine proteases[71]. Rivaroxaban inhibits prothrombinase (IC_{50} of 2.1 nM)[71] and initial data suggest that it also inhibits clot-bound factor Xa activity (IC_{50} of 75 nM)[72]. In human plasma,

Deep-vein thrombosis
(DVT). A blood clot in a deep vein, usually in the leg. Distal DVT occurs in the calf, whereas proximal DVT occurs above the knee.

Pulmonary embolism
A blood clot or thromboembolus in a pulmonary blood vessel. Such emboli generally originate from a deep-vein thrombosis and can cause permanent lung damage, chronic pulmonary hypertension and death.

Haemostasis
The complex process that leads to the formation of a blood clot, causing bleeding to stop.

Box 1 | **Binding mode of rivaroxaban**



The figure shows the X-ray crystal structure of rivaroxaban (carbons coloured orange) in complex with human factor Xa[63]. Essential amino acids and binding pockets (S1 and S4) are indicated; hydrogen bonds are shown as dotted lines.

Two hydrogen bonds are formed between rivaroxaban and the amino acid Gly219 of factor Xa. The first, a strong interaction (2.0 Å), is from the carbonyl oxygen of the oxazolidinone core. The second, weaker interaction (3.3 Å), is from the amino group of the chlorothiophene carboxamide moiety. These two hydrogen bonds support the oxazolidinone core in directing its substituents into the S1 and the S4 subsites of factor Xa. This results in rivaroxaban forming an L-shape, a binding mode typical of synthetic, direct factor Xa inhibitors[63].

The aromatic rings of Tyr99, Phe174 and Trp215 in factor Xa define a narrow hydrophobic channel that comprises the S4 pocket. The morpholinone moiety of rivaroxaban is 'sandwiched' between Tyr99 and Phe174, while its aryl ring is oriented perpendicularly, extending across the face of Trp215. There is no direct interaction between the morpholinone carbonyl group and the factor Xa backbone; rather, this carbonyl group contributes to a planarization of the morpholinone ring, further supporting the sandwich-like arrangement[63]. Using the morpholinone moiety, as well as other six-membered rings such as lactames or pyridinones, we found new, non-basic P4 residues (see REF. 146 for patent application), yielding strongly increased binding to factor Xa. Such residues have since been used successfully for several other factor Xa inhibitors[69,147], including apixaban[148] and PD0348292 (eribaxaban)[149].

In the S1 pocket of factor Xa, the key interaction is between the chlorine substituent of the thiophene moiety and the aromatic ring of Tyr228 at the bottom of the S1 pocket. This novel interaction obviates the need for strongly basic groups, such as amidines, to achieve high factor Xa affinity, and therefore enables non-basic rivaroxaban to achieve both high potency and good oral bioavailability[63]. A similar binding mode has been found and reported for several other factor Xa inhibitors[66–70].

Figure modified, with permission, from REF. 63 © (2005) American Chemical Society.

Fibrinogen
A soluble plasma protein that, in the final phase of the coagulation process, is converted to fibrin by thrombin. Fibrin then polymerizes and forms the fibrous network base of a clot.

Oral bioavailability
The total proportion of pharmacologically active drug that enters the systemic circulation after oral administration. It is affected by both absorption and local metabolic inactivation.

Prothrombin time
A laboratory test that measures clotting time in the presence of tissue factor (thromboplastin). It is used to assess the activity of the extrinsic coagulation pathway.

Activated partial thromboplastin time
A laboratory test that measures the clotting time of plasma after contact activation. It assesses the function of the intrinsic coagulation pathway.

rivaroxaban inhibited thrombin generation — and therefore the amplification processes of coagulation — through the inhibition of factor Xa generated by either the intrinsic or the extrinsic coagulation pathways[73,74]. Thrombin generation was almost completely inhibited in platelet-rich plasma at physiologically relevant concentrations (80–100 nM) of rivaroxaban[73]. This and other studies demonstrated that rivaroxaban prolonged the initiation phase of thrombin generation, potently inhibited the physiologically relevant prothrombinase complex-bound factor Xa on the surface of activated platelets[71] and reduced the thrombin burst produced in the propagation phase[73].

In addition, preliminary work has shown that rivaroxaban inhibits thrombin generation in the presence and absence of thrombomodulin in human plasma in a concentration-dependent manner. This suggests that it does not interfere with the thrombin–thrombomodulin–APC system and, therefore, probably does not suppress APC-mediated negative feedback[75], as was shown for DX-9065a[49]. Further preliminary data suggested that rivaroxaban did not directly affect platelet aggregation in platelet-rich plasma induced by thrombin, adenosine diphosphate or collagen[76], but potently inhibited tissue-factor-induced platelet aggregation in an indirect manner, by inhibiting thrombin generation[77].

Rivaroxaban demonstrated anticoagulant effects in human plasma, with the prothrombin time being more sensitive than the activated partial thromboplastin time[71], a finding also observed with other direct factor Xa inhibitors in clinical development[78,79]. This may be because direct factor Xa inhibitors, including rivaroxaban[71], are highly effective inhibitors of the prothrombinase complex, although differences in enzyme kinetics may also be responsible[20]. A dose-dependent prolongation of prothrombin time was demonstrated *in vivo* in rat and rabbit models, with a strong correlation observed between prothrombin time and plasma concentrations of rivaroxaban (r=0.98)[71].

In vivo, rivaroxaban given prophylactically had potent and consistent antithrombotic effects in venous[71,80] and arterial[71] thrombosis models in rats and rabbits. In a rabbit treatment model, rivaroxaban reduced the accretion of radiolabelled fibrinogen into preformed clots in the jugular vein, relative to untreated controls[80]. Bleeding times in rats and rabbits were not significantly affected at antithrombotic-effective doses[71], indicating a favourable efficacy/bleeding ratio.

Although rivaroxaban has a short half-life in humans[81,82] (see the Xarelto Summary of Product Characteristics (SMPC) from the European Medicines

Agency (EMA); Further information), it may be of interest to determine whether the anticoagulant effect of rivaroxaban can be reversed, because there might be rare instances in which this would be of use — for example, a need for emergency surgery. However, it is worth noting that several established anticoagulants also lack full antidotes. For example, the anticoagulant effect of the LMWHs is only partly reversed by protamine sulphate[2] and there is no available antidote for fondaparinux (see Arixtra's prescribing information; Further information). Preliminary studies in primates and rats have been conducted to evaluate possible antidotes for rivaroxaban. These studies suggested that recombinant factor VIIa, activated prothrombin complex concentrate (FEIBA (factor VIII inhibitor bypassing activity); Baxter)[83] and prothrombin complex concentrate[84] can partially reverse, in a dose-dependent manner, the effects of high-dose rivaroxaban on bleeding time. However, it is important to note that there is no clinical experience with any of these reversal strategies (see the EMA's SMPC on rivaroxaban; Further information).

Clinical pharmacology

In Phase I and Phase II studies, rivaroxaban exhibited predictable pharmacokinetic and pharmacodynamic profiles, as follows. It is rapidly absorbed, with maximum plasma concentrations (C_{max}) occurring 2–4 hours after tablet intake[82,85]. Oral bioavailability of rivaroxaban is decreased at higher doses, possibly owing to poor solubility. However, at the currently approved 10 mg dose, oral bioavailability is 80–100%[85].

Food (a high-fat, high-calorie meal) did not affect the area under the plasma concentration–time curve (AUC) or C_{max} for the 10 mg tablet (EMA's SMPC on rivaroxaban). Rivaroxaban was safe and well tolerated across a wide dose range, with dose-proportional pharmacokinetics and pharmacodynamics. No relevant accumulation was observed at any dose level after multiple dosing in healthy subjects at steady state[82].

Rivaroxaban is metabolized by CYP450 isoforms, particularly CYP3A4, which is strongly inhibited by ketoconazole and ritonavir (see prescribing information from Janssen Pharmaceutica (ketoconazole) and Abbott (ritonavir), and the EMA's Committee of Medicinal Products for Human Use (CHMP) report for rivaroxaban; Further information). These drugs were, therefore, predicted to affect the metabolism of rivaroxaban and, because both drugs are also strong inhibitors of P-glycoprotein (P-gp) and CYP3A4 (REFS 86,87), may increase plasma concentrations of rivaroxaban. This expectation was confirmed in subsequent Phase I studies that showed a strong interaction between rivaroxaban and these two drugs. However, there was no clinically relevant interaction with clarithromycin, a strong CYP3A4 inhibitor but only a moderate inhibitor of P-gp, indicating that rivaroxaban may be used with substances that strongly inhibit only one of the two elimination pathways (see the EMA's SMPC on rivaroxaban and prescribing information from Janssen Pharmaceutica (ketoconazole) and Abbott (ritonavir)). Conversely, these findings show that concomitant use of rivaroxaban with strong inhibitors of both CYP3A4 and P-gp such as ketoconazole or HIV protease inhibitors such as ritonavir is not recommended. In addition, rivaroxaban should be used with caution if strong CYP3A4 inducers are administered concomitantly (EMA's CHMP report for rivaroxaban).

Rivaroxaban has a low propensity for drug–drug interactions with frequently used concomitant medications such as naproxen[88] and asprin[89], and no interaction with the cardiac glycoside digoxin (Lanoxin; GlaxoSmithKline) (D. Kubitza, unpublished observations).

Furthermore, dietary restrictions are not necessary at the 10 mg once-daily dose; rivaroxaban was given with and without food in the Phase III VTE-prevention studies (RECORD studies 1–4)[90–93] (EMA's SMPC on rivaroxaban).

Rivaroxaban is distributed heterogeneously to tissues and organs, and exhibits only moderate tissue affinity in rats; importantly, it does not substantially penetrate the blood–brain barrier[94]. However, like many small molecules, rivaroxaban is expected to be able to cross the placenta, although specific studies have not been published. *In vitro* and Phase I studies showed that rivaroxaban has a dual mode of elimination, with one-third eliminated unchanged by the kidneys and two-thirds metabolized by the liver to inactive metabolites, with no major or active circulating metabolites detected in plasma[95,96]. Elimination of rivaroxaban from plasma occurs with a mean terminal half-life of 7–11 hours[81,82] (EMA's SMPC on rivaroxaban). With a systemic clearance rate of approximately 10 L h^{-1}, rivaroxaban can be classified as a low-clearance drug[97,98]. Low intra-individual and moderate inter-individual pharmacokinetic variability have been observed[97,98]. In Phase I studies, the coefficient of variation for AUC ranged from 18% to 33%, and for C_{max} from 16% to 39%, for inter-individual variability. Median intra-individual variability was only 14% and 19% for AUC and C_{max}, respectively (EMA's CHMP report for rivaroxaban).

Rivaroxaban inhibited factor Xa activity in a dose-dependent manner after single dosing in healthy subjects. Rivaroxaban also inhibited thrombin generation in healthy subjects, and some parameters of thrombin generation remained inhibited for 24 hours after administration of a 30 mg dose[74]. These results offered the first indication that once-daily dosing might be feasible.

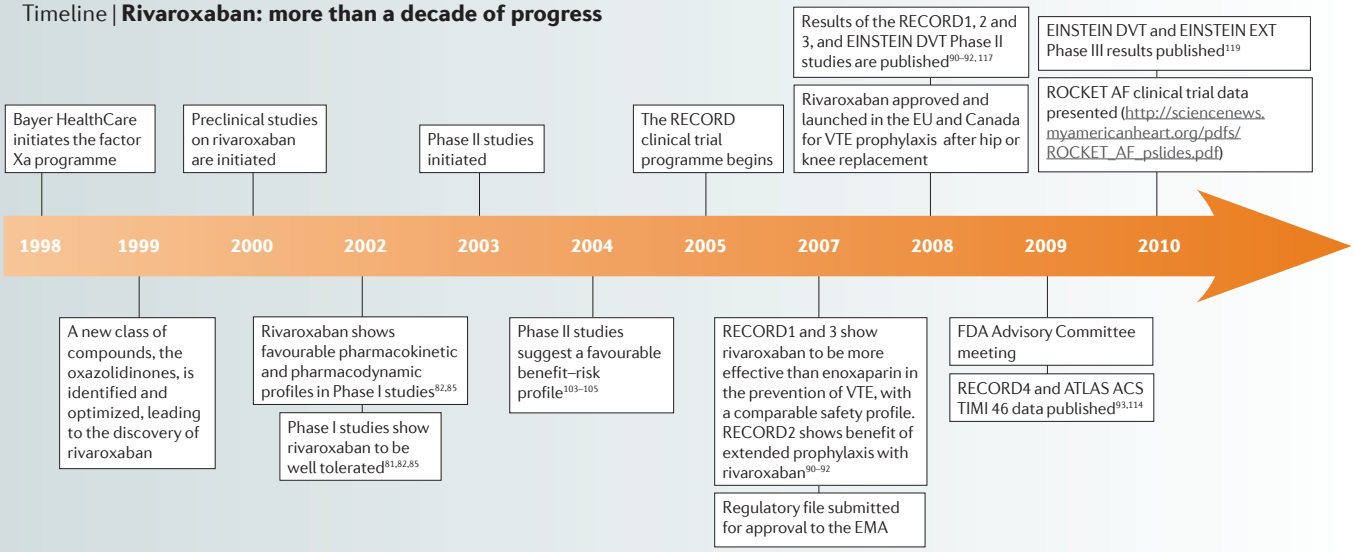
There were close correlations between pharmacokinetic and pharmacodynamic parameters[82]. Rivaroxaban plasma concentrations correlated closely with prolongation of prothrombin time and inhibition of factor Xa. Plasma concentrations correlated with prothrombin time both in healthy volunteers[82] and in patients undergoing either total hip replacement (THR) or total knee replacement (TKR) in Phase II studies[97].

Various Phase I and II studies, using total daily doses ranging from 5 mg to 80 mg, indicated that rivaroxaban could be given irrespective of age, gender[81], body weight[99] and mild (creatinine clearance: 50–79 ml min^{-1}) to moderate (creatinine clearance: 30–49 ml min^{-1}) renal impairment[100]. A further preliminary clinical study suggested that rivaroxaban can also be given irrespective of mild hepatic impairment (classified as Child–Pugh class A)[101]. Fixed doses of rivaroxaban were administered

CYP450 isoforms
Many therapeutic drugs are metabolized by cytochrome P450 (CYP450) enzymes, a 'superfamily' of related but distinct enzymes that differ in their substrate specificity.

Creatinine clearance
The rate at which the kidney clears the blood of creatinine (a waste product from muscles that is excreted at a fairly constant rate). Creatinine clearance is used as an approximation of the glomerular filtration rate.

Timeline | **Rivaroxaban: more than a decade of progress**



DVT, deep-vein thrombosis; EMA, European Medicines Agency; EU, European Union; FDA, US Food and Drug Administration; VTE, venous thromboembolism.

in Phase II studies investigating rivaroxaban for the prevention or treatment of VTE, with no restrictions on gender, or mild or moderate renal impairment[102–105]. These parameters were shown to have no clinically relevant effect on the pharmacokinetics and pharmacodynamics of rivaroxaban[97]. In addition, rivaroxaban had no effect on heart-rate-corrected QT interval prolongation[106]. Owing to its pharmacokinetic and pharmacodynamic profiles, rivaroxaban can be given at fixed doses to adult patients with no requirement for routine coagulation monitoring. However, there may be rare occasions when monitoring is of use — for example, where poor compliance is suspected or emergency surgery is required, or to confirm a possible overdose. This concern has led to the evaluation of a number of different assay types. Initial results suggest that, in light of an apparent strong correlation between the extent of factor Xa inhibition and rivaroxaban plasma concentration, chromogenic assays for factor Xa may be particularly suited for the quantification of rivaroxaban in plasma[107,108].

Clinical development of rivaroxaban

Rivaroxaban is approved for the prevention of VTE after elective hip or knee replacement in approximately 100 countries, including member states of the European Union (as of 30 September 2008) and Canada (as of 16 September 2008). Clinical development is ongoing for the prevention and treatment of thromboembolic disorders in other conditions, including the treatment of VTE, the prevention of VTE in hospitalized, medically ill patients (for example, patients hospitalized with an acute medical condition, such as cancer, heart failure or respiratory failure, or requiring intensive care), as well as the prevention of stroke in patients with atrial fibrillation and secondary prevention in patients with ACS. The TIMELINE highlights the development of rivaroxaban during the past decade.

VTE prophylaxis after total hip or knee replacement. A large, comprehensive Phase II programme involving about 2,900 patients assessed both once-daily and twice-daily dosing regimens[102–105]. Collectively, these studies demonstrated an optimal dose range of 5–20 mg per day[109], and indicated that rivaroxaban 10 mg once daily had the optimum balance of efficacy and safety, relative to enoxaparin 40 mg once daily[103] (FIG. 5).

On the basis of the results of the Phase II studies, rivaroxaban 10 mg once daily was selected for investigation in the Phase III RECORD programme, which comprised four large studies involving a total of more than 12,500 patients undergoing elective THR or TKR[90–93] (TABLE 2). In all four studies, the primary efficacy end point was the composite of any DVT, as detected by mandatory, bilateral venography, non-fatal pulmonary embolism and all-cause mortality. The primary safety end point was major bleeding, which in the RECORD programme did not include surgical-site bleeding[90–93].

The RECORD1 and 3 studies were designed to compare rivaroxaban 10 mg once daily with the standard of care — enoxaparin 40 mg once daily — both given for 31–39 days (extended prophylaxis) after THR (RECORD1)[90] or for 10–14 days after TKR (RECORD3)[92]. In both studies, rivaroxaban was more effective than enoxaparin for the prevention of VTE[90,92] (TABLE 2). Furthermore, the incidence of major bleeding was comparable and not significantly different between treatment groups[90,92]. RECORD3 also showed a significant reduction in symptomatic VTE for rivaroxaban[92].

RECORD2 investigated the efficacy and safety of extended thromboprophylaxis with rivaroxaban (35 days; range 31–39 days) compared with short-term enoxaparin treatment (10–14 days) followed by placebo in patients undergoing THR[91]. The results demonstrated that extended prophylaxis with rivaroxaban (10 mg once daily) was superior to short-term prophylaxis with enoxaparin (40 mg once daily) followed by placebo for

Chromogenic assay
An enzymatic assay in which a colour develops during the course of the reaction, which can then be measured spectrophotometrically. Colour development is reduced in the presence of an inhibitor.

Venography
Radiography of the veins after intravenous injection of a radioactive isotope or contrast dye. This can be used to confirm the presence of deep-vein thromboses.

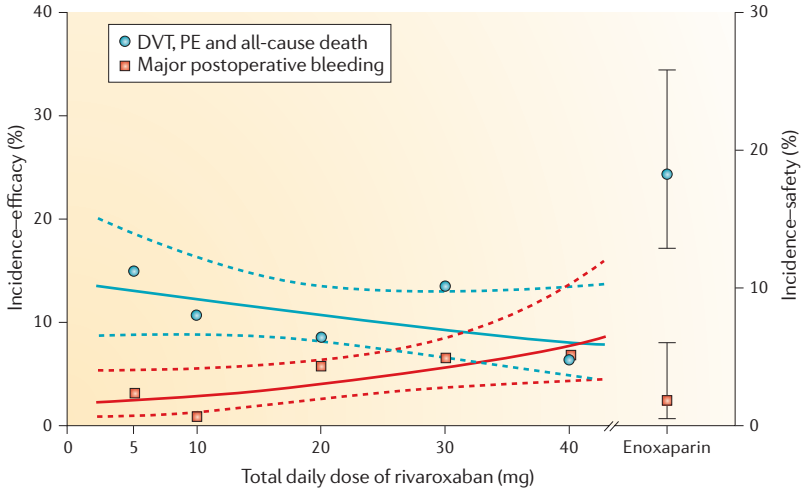


Figure 5 | **Efficacy and safety dose–response relationships.** The figure shows the dose–response relationships between rivaroxaban total daily dose and the primary efficacy end point (any deep-vein thrombosis (DVT); non-fatal pulmonary embolism (PE) and all-cause death) and primary safety end point (major bleeding) in the once-daily study investigating rivaroxaban for the prevention of venous thromboembolism after total hip replacement[103]. Solid lines show the dose–response curves (logistic regression), blue hatched lines represent 95% confidence intervals for efficacy and red hatched lines represent 95% confidence intervals for safety. Details for 40 mg enoxaparin once daily, the current European standard of care, are shown for comparison. Figure reproduced, with permission, from REF. 103 © (2006) Lippincott Williams & Wilkins.

the prevention of VTE, including symptomatic events[91] (TABLE 2). Despite rivaroxaban being given for 3 weeks longer than enoxaparin, the incidence of treatment-emergent major bleeding (that is, up to 2 days after the last dose of study medication) was <0.1% in both treatment groups. Guidelines recommend a minimum duration for prophylaxis of 10 days, and also recommend that prophylaxis be extended for up to 35 days for patients undergoing THR[110]. The RECORD2 study provided additional confirmation of the benefits of extended prophylaxis over short-term prophylaxis after THR.

RECORD4 compared the efficacy and safety of oral rivaroxaban 10 mg once daily with the commonly used North American regimen of enoxaparin 30 mg twice daily in patients undergoing TKR[93]. Rivaroxaban was significantly superior to enoxaparin for the primary efficacy end point, with no significant difference in the rates of major bleeding between the two groups (TABLE 2). So far, rivaroxaban is the only new oral anticoagulant to demonstrate superior efficacy over the greater dose intensity of the North American enoxaparin regimen[93,111,112].

A comparison of rivaroxaban with enoxaparin in these four studies showed the efficacy and safety of rivaroxaban in the prevention of VTE in patients undergoing elective THR or TKR. The superiority of rivaroxaban for the primary efficacy end point was demonstrated in all four studies. Rivaroxaban also showed a good safety profile, with a low incidence of major bleeding, comparable to that observed with enoxaparin[90–93], and no evidence of compromised liver function attributable to rivaroxaban[113]. Furthermore, the incidence of haemorrhagic wound complications (composite of excessive wound haematoma and reported surgical-site bleeding) was similar in both treatment groups[113]. Liver safety is also an important consideration in regulatory review. In a Phase II trial of rivaroxaban in patients with ACS, no patient who had received rivaroxaban for 6 months had an alanine-amino-transferase level greater than three times the upper limit of normal or total bilirubin greater than twice the upper limit of normal[114].

These studies were also conducted with no routine coagulation monitoring and no dose adjustment for demographic variables, consistent with preliminary results from pooled subgroup analyses[115].

Treatment of VTE. The efficacy and safety of rivaroxaban for the treatment of VTE were assessed in two Phase IIb dose-ranging studies[116,117]. These studies suggested that rivaroxaban had good efficacy and a similar safety profile, compared with standard therapy, for the treatment of acute symptomatic DVT. An initial intensified twice-daily regimen (rivaroxaban 15 mg twice daily for 3 weeks) followed by convenient 20 mg once-daily dosing for 3, 6 or 12 months was selected for investigation in Phase III studies. The efficacy and safety of rivaroxaban for the treatment of VTE are being assessed in three Phase III studies involving approximately 9,000 patients in total— EINSTEIN DVT (ClinicalTrials.gov identifier: NCT00440193), EINSTEIN PE (NCT00439777) and EINSTEIN EXT (NCT00439725).

EINSTEIN PE (pulmonary embolism) is still ongoing. However, data for EINSTEIN DVT (presented at the 2010 American Society of Hematology meeting[118] and recently published[119]) showed that rivaroxaban (15 mg twice daily for 21 days followed by 20 mg once daily) was non-inferior for the prevention of recurrent symptomatic VTE in comparison to the current standard of care (enoxaparin 1.0 mg kg^{-1} twice daily for ≥5 days followed by VKA titrated to INR 2.0–3.0). First symptomatic recurrent VTEs occurred in 2.1% of patients receiving rivaroxaban compared with 3.0% of those in the enoxaparin/VKA treatment arm. Major or non-major clinically relevant bleeding occurred in 8.1% of patients in each treatment arm[119].

In the EINSTEIN EXT trial, 1,196 patients (intention-to-treat population) who had completed 6–12 months of anticoagulant therapy for the acute index event (VTE) were randomized to an additional 6–12 months of therapy with either rivaroxaban, 20 mg once daily, or placebo[119]. Study medication was administered for a mean period of 190 days in each treatment group. Recurrent symptomatic VTE events were observed in 7.1% and 1.3% of the placebo and rivaroxaban treatment groups, respectively (hazard ratio 0.18, $P<0.0001$), a relative risk reduction of 82% with rivaroxaban[119] (hazard ratio, 0.68; 95% confidence interval, 0.44–11.04; P<0.001 for non-inferiority). Major bleeding did not occur in any placebo-treated patients and occurred in four (0.7%) rivaroxaban-treated patients, although none of these occurred in a critical location or proved fatal[119].

Thromboprophylaxis in medically ill patients. A Phase III study (NCT00571649) has been initiated to investigate the efficacy and safety of VTE prophylaxis

Index event
The acute event that leads to a patient's initial presentation. The term can also refer to the initial event resulting in a patient's inclusion in a follow-up study, such as a survey of recurrent strokes.

Table 2 | **Incidence of venous thromboembolism and bleeding events across the four RECORD* studies**

End points‡	Efficacy end point (% (*n*/*N*))			Safety end points (patients with bleeding events) (% (*n*/*N*))	
	Total VTE	Major VTE	Symptomatic VTE	Major bleeding	Major and non-major clinically relevant bleeding
RECORD1 (THR: prophylaxis administered for 35 days)					
Enoxaparin (40 mg once daily)	3.7 (58/1,558)	2.0 (33/1,678)	0.5 (11/2,206)	0.1 (2/2,224)	2.5 (56/2,224)
Rivaroxaban (10 mg once daily)	1.1 (18/1,595)	0.2 (4/1,686)	0.3 (6/2,193)	0.3 (6/2,209)	3.2 (70/2,209)
P value	<0.001	<0.001	0.22	0.18	NS§
RECORD2 (THR: extended rivaroxaban prophylaxis, 35 days, versus short-term enoxaparin, 14 days, followed by placebo)					
Enoxaparin (40 mg once daily/placebo)	9.3 (81/869)	5.1 (49/962)	1.2 (15/1,207)	<0.1 (1/1,229)	2.8 (34/1,229)
Rivaroxaban (10 mg once daily)	2.0 (17/864)	0.6 (6/961)	0.2 (3/1,212)	<0.1 (1/1,228)	3.3 (41/1,228)
P value	<0.0001	<0.0001	0.004	0.98 (REF. 157)	NR
RECORD3 (TKR: prophylaxis administered for 14 days)					
Enoxaparin (40 mg once daily)	18.9 (166/878)	2.6 (24/925)	2.0 (24/1,217)	0.5 (6/1,239)	2.7 (34/1,239)
Rivaroxaban (10 mg once daily)	9.6 (79/824)	1.0 (9/908)	0.7 (8/1,201)	0.6 (7/1,220)	3.3 (40/1,220)
P value	<0.001	0.01	0.005	0.77	0.44
RECORD4 (TKR: prophylaxis administered for 14 days)					
Enoxaparin (30 mg twice daily)	10.1 (97/959)	2.0 (22/1,112)	1.2 (18/1,508)	0.3 (4/1,508)	2.3 (34/1,508)
Rivaroxaban (10 mg once daily)	6.9 (67/965)	1.2 (13/1,112)	0.7 (11/1,526)	0.7 (10/1,526)	3.0 (46/1,526)
P value	0.012	0.124	0.187	0.11	0.18

N, total number of patients evaluable in each treatment group; NR, not reported; NS, not significant; THR, total hip replacement; TKR, total knee replacement; VTE, venous thromboembolism. *The four RECORD studies[90–93] compared rivaroxaban regimens with enoxaparin regimens (the current standard of care); summary results are shown in the table. ‡Results shown as number (*n*) of patients experiencing an event; patients could have more than one event. §*P* value not reported, but 95% confidence interval for risk difference includes zero.

with rivaroxaban 10 mg once daily (for 35 ± 4 days), compared with short-term 40 mg once-daily enoxaparin (administered for 10 ± 4 days followed by placebo), in hospitalized, medically ill patients.

Stroke prevention in non-valvular atrial fibrillation. Rivaroxaban 20 mg once daily has been compared with warfarin for the prevention of stroke and non-central-nervous-system systemic embolism in approximately 14,000 patients with non-valvular atrial fibrillation in a Phase III study (NCT00403767)[120]. Patients with moderate renal impairment (creatinine clearance: 30–49 ml min^{-1}) received a fixed dose of 15 mg once daily. It was recently reported at the 2010 American Heart Association meeting that, in the ROCKET AF study, rivaroxaban significantly reduced the risk of stroke and non-central-nervous-system thromboembolism in patients with atrial fibrillation compared to warfarin, with comparable rates of bleeding (http://sciencenews.myamericanheart.org/pdfs/ROCKET_AF_pslides.pdf).

Secondary prevention in ACS. A Phase III study investigating secondary prevention of ischaemic events in patients with ACS (NCT00809965) was started in late 2008, and is expected to enrol up to 16,000 patients. Two doses of rivaroxaban, 2.5 mg and 5 mg twice daily, are being investigated on the basis of the results of a Phase IIb study that assessed safety and efficacy in approximately 3,500 patients with recent, non-ST-elevation myocardial infarction, ST-elevation myocardial infarction or unstable angina[114]. As noted above, this trial also showed no evidence of compromised liver function in patients receiving rivaroxaban for up to 6 months.

Other direct factor Xa inhibitors in development

Below, we discuss other direct factor Xa inhibitors that are in advanced clinical development (Phase III).

Apixaban. Apixaban (developed by Pfizer/Bristol-Myers Squibb) is a small-molecule, oral, direct factor Xa inhibitor (FIG. 3) that selectively and reversibly inhibits both free factor Xa (K_i of 0.08 nM) and prothrombinase activity[79,121]. Another study reported that apixaban reacts rapidly with factor Xa (k_{on} 2×10^7 M^{-1} s^{-1}) with high-affinity binding (K_i of 0.3 nM at 37 °C)[122]. Preclinical studies have shown that apixaban was well absorbed in chimpanzees, dogs and rats; mean oral bioavailability was 51% (chimpanzees), 88% (dogs) and 34% (rats)[79]. The drug is currently

being evaluated in a number of thromboembolic indications, including the prevention and treatment of VTE, the prevention of stroke in patients with atrial fibrillation and the prevention of cardiovascular events in ACS.

In the Phase III programme for the prevention of VTE after major orthopaedic surgery, apixaban did not meet the prespecified criteria for non-inferiority compared with enoxaparin 30 mg twice daily (NCT00371683) for VTE prevention after TKR[111]. However, a second study (NCT00452530) conducted in patients undergoing TKR demonstrated superior efficacy for apixaban against enoxaparin 40 mg once daily. Clinically relevant bleeding (major or non-major) occurred in fewer patients given apixaban, although the differences were not significant[123]. A third study (NCT00423319), presented as an abstract, assessed extended prophylaxis with apixaban versus enoxaparin (40 mg once daily) in patients undergoing THR and also demonstrated superior efficacy for apixaban with similar rates of major and non-major clinically relevant bleeding[124].

A Phase II trial in patients with ACS (NCT00313300) assessed efficacy and safety in patients taking apixaban compared with placebo[125]. The developing companies recently reported that a subsequent Phase III trial (NCT00831441) in patients with ACS investigating whether apixaban (5 mg twice daily) is superior to placebo has been halted (press release, Bristol-Myers Squibb; Further information). In addition, other apixaban trials are ongoing for VTE prevention in patients with acute medical illness (NCT00457002), or recently completed for malignant disease (NCT00320255), as well as Phase III trials for the treatment of VTE (NCT00633893, NCT00643201) and trials for the prevention of stroke in patients with atrial fibrillation (NCT00412984, NCT00496769)[126,127]. Data for the AVERROES trial (NCT00496769) were recently presented at the European Society of Cardiology Congress. Patients with atrial fibrillation who have either been demonstrated to be or were expected to be unsuitable for treatment with VKAs received apixaban 5 mg twice daily or aspirin 81–324 mg per day. Preliminary results showed that the primary end point of stroke or systemic embolism occurred at a significantly lower rate in patients receiving apixaban, with an absolute risk reduction of approximately 2% versus aspirin[128].

Edoxaban. Edoxaban (developed by Daiichi Sankyo) is an oral, direct and specific factor Xa inhibitor with a K_i of 0.56 nM (FIG. 3); K_i values for other coagulation factors are >10,000-fold higher[78]. In healthy volunteers, peak plasma levels of edoxaban were observed at 1.5 hours after a single oral dose, corresponding to the maximum inhibition of factor Xa activity; and *ex vivo* thrombus formation was reduced at 1.5 hours and 5 hours after administration[129].

Phase II trials in patients undergoing TKR[130] or THR[131] have been completed, as well as a Phase II trial for stroke prevention in atrial fibrillation[132], and two Phase III trials are now under way. One is designed to compare two different doses of edoxaban with warfarin for stroke prevention in patients with atrial fibrillation (NCT00781391) and another is evaluating edoxaban for the secondary prevention of recurrent VTE in patients with acute symptomatic proximal DVT or pulmonary embolism (NCT00986154).

YM150. YM150 (developed by Astellas) is also an oral, direct factor Xa inhibitor (K_i of 31 nM). Preliminary data show that both YM150 and its major metabolite YM-222714 (K_i of 20 nM) have antithrombotic effects at doses that do not prolong template bleeding time in animal models of venous and arterial thrombosis[133]. Phase II trials for VTE prevention after THR[134] or TKR (NCT00408239) have been completed, as has a warfarin-controlled Phase II trial for stroke prevention in patients with atrial fibrillation (NCT00448214). Three Phase III trials are now in progress. One will assess once-daily and twice-daily doses of YM150 against enoxaparin for VTE prevention in patients undergoing elective hip replacement (NCT00902928). In another Phase III trial, YM150 is being compared with mechanical prophylaxis for the prevention of VTE after major abdominal surgery (NCT00942435). A third study is being conducted in Japan to evaluate YM150 for the prevention of VTE in the 28 days following hospitalization for an acute medical illness (NCT01028950).

Outlook

For more than 65 years, VKAs have been the only available oral anticoagulants, and although effective, the need for dose adjustment and periodic coagulation monitoring considerably complicates their clinical management. New and improved anticoagulants could potentially address the shortcomings associated with the current standard of care and it should be noted that other approaches, besides the development of oral, direct factor Xa inhibitors, are also being evaluated. For example, the parenteral direct factor Xa inhibitor, otamixaban (developed by Sanofi–Aventis), has completed Phase II trials for short-term use in non-ST-elevation ACS[135] and in percutaneous coronary intervention[136]. The encouraging results obtained in these studies have been followed by an ongoing Phase III trial in patients with unstable angina or non-ST elevation myocardial infarction undergoing an invasive intervention (NCT01076764). Conversely, idraparinux, and its successor idrabiotaparinux[137] (Sanofi–Aventis; both now discontinued) are parenterally administered pentasaccharides (indirect factor Xa inhibitors) with very long half-lives[138] that were developed to permit once-weekly dosing in indications requiring long-term or chronic therapy[139,140].

In addition, direct thrombin inhibitors are also in development, most notably dabigatran (Pradaxa/Pradax; Boehringer Ingelheim), which, like rivaroxaban, has completed several Phase III thrombosis prevention and treatment studies[141–144] and has been approved in member states of the European Union and other countries for the prevention of VTE after elective hip or knee replacement. Dabigatran is also in trials for a number of other indications, as is AZD0837 (developed by AstraZeneca), which is in Phase II[145]. Although indirect comparisons, based on meta-analyses, can be conducted, direct comparative trials are required for a comprehensive evaluation of one particular drug regimen versus another.

In the future, it is anticipated that long-term anticoagulant therapy will favour oral agents with a wide therapeutic window and a predictable anticoagulant response that do not require routine coagulation monitoring or dose adjustment. Emerging data suggest that direct factor Xa inhibitors are both effective antithrombotic agents for short-term use and promising agents for long-term usage. As they have shown a predictable pharmacological profile, are given orally and do not require routine coagulation monitoring, these new agents may also facilitate patient compliance and adherence to clinical guidelines. Thus, they are likely to improve anticoagulation treatment in thromboembolic disorders and reduce the burden associated with long-term therapy, thereby providing important alternative options for the management of these conditions.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

FURTHER INFORMATION
ClinicalTrials.gov: http://clinicaltrials.gov
Bayer HealthCare Xarelto (rivaroxaban) Summary of Product Characteristics: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000944/WC500057108.pdf
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