OPINION

Targeting tumour-supportive cellular machineries in anticancer drug development

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Abstract | Traditional anticancer chemotherapeutics targeting DNA replication and cell division have severe side effects, but they have proved to be highly successful in treating some cancers. Drugs targeting signalling oncoproteins that have gained tumour-driving functions through mutations or overexpression were subsequently developed to increase specificity and thus reduce side effects, but have limitations such as the development of resistance. Now, a new wave of small-molecule anticancer agents is emerging, targeting complex multicomponent cellular machineries — including chromatin modifiers, heat shock protein chaperones and the proteasome — which thus interfere with those support systems that are more essential for cancer cells than for normal cells. Here, we provide our perspective on the advantages and limitations of agents that target tumour-supportive cellular machineries (other than those involving DNA replication), comparing them with agents that target signalling intermediates.

Be uncomfortable; be sand, not oil,
in the machinery of the world.
Günter Eich (German poet, 1907–1972)

The history of anticancer drug development can be broadly divided into three 'epochs' or 'waves' that emerged sequentially (FIG. 1; TIMELINE). In the first wave, drugs mainly blocked DNA replication and cell division — for example, by modifying DNA or by interfering with tubulin associations. In the second wave, drugs targeted signalling intermediates that contribute to cancer growth, most notably receptors and/or kinases such as BCR–ABL or BRAF. Now, in the third wave, drugs are being developed that target cellular machineries that are not directly involved in DNA replication or cell division but are essential for tumour growth and survival, such as chromatin modifiers, protein chaperones or the proteasome. Drugs that target such cellular machineries are already on the market — such as the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals) for multiple myeloma — but a growing body of research has identified additional cellular multisubunit complexes that are more essential for the growth and survival of cancer cells than normal cells, such as the machineries driving RNA and protein synthesis, intracellular transport, metabolic pathways and the integrity of organelles.

This article first provides a brief overview of what has been achieved by the pioneering drugs of the first and second waves of anticancer drugs, as well as their limitations. Then, we explore the emerging agents that are shaping the third wave of anticancer drugs, which aim to exploit the added cellular stresses that are associated with tumours (for example, proteotoxic stress or dysregulated chromatin dynamics). Finally, we evaluate each wave of anticancer drugs, highlighting the possibilities of harnessing the advantages of each category to create combinations that could overcome the limitations of existing treatments. Although this article focuses on small molecules, discussions on monoclonal antibodies and antibody–drug conjugates (ADCs) are provided where appropriate.

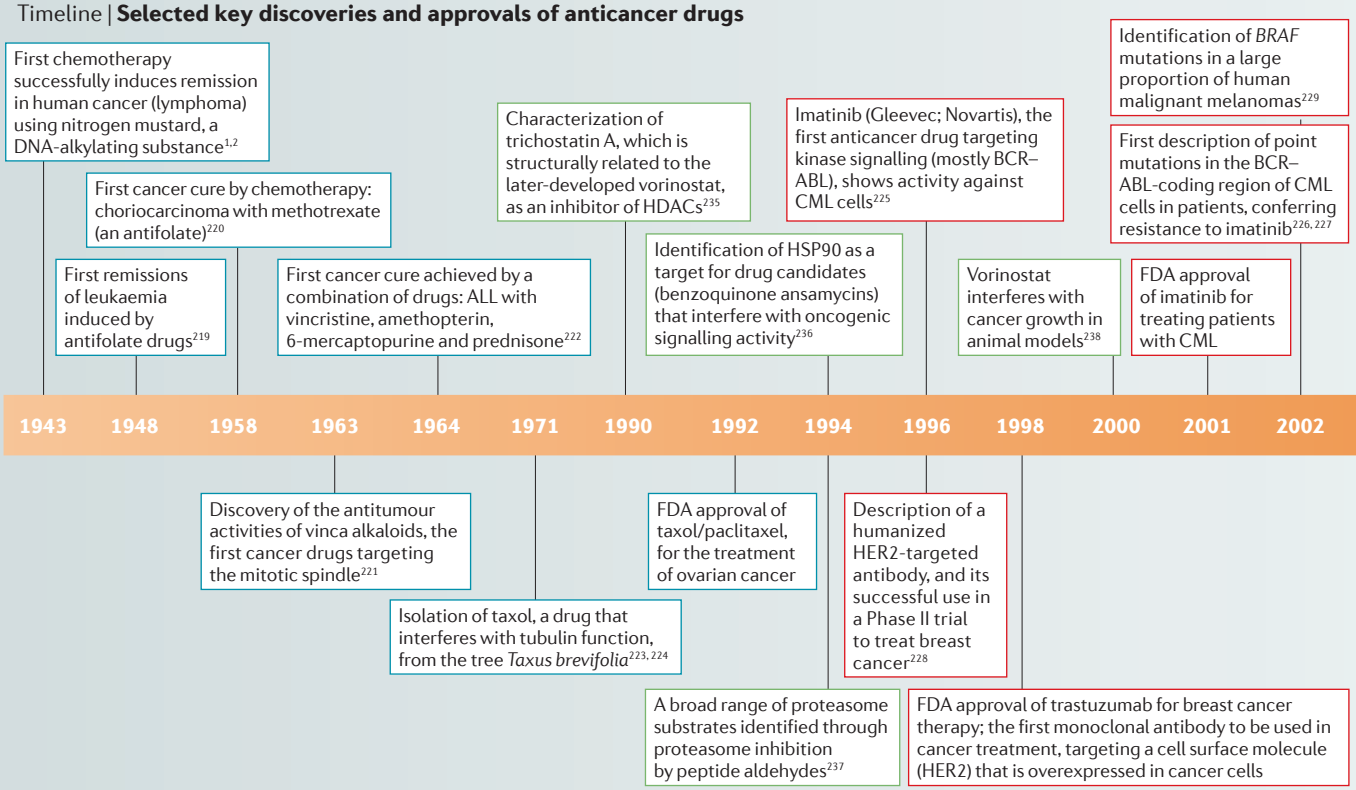
First-wave anticancer drugs

The first-wave anticancer drugs (that is, chemotherapeutics) were identified either through accidental observations or on the basis of their similarity to essential cofactors in haematopoiesis. For example, the first DNA-modifying chemotherapeutic was derived from mustard gas; survivors of mustard gas exposure resulting from warfare suffered from leukopaenia, and this led to the use of the mustard gas derivative nitrogen mustard in lymphoma treatment in 1943 (REFS 1,2) (FIG. 1; TIMELINE).

For many decades in the twentieth century, the use of such drugs (their dosage, timing and regimen) was refined based on purely clinical observations[3]. Only slowly did it become clear that many of these chemotherapeutics act by interfering with the integrity and/or replication of DNA, or that they block mitosis by interfering with the microtubule dynamics of the mitotic spindle[4]. These early anticancer drugs — for example, platinum derivatives, topoisomerase inhibitors, nucleoside analogues, vinca alkaloids and taxanes — still represent the vast majority of clinically used chemotherapeutics today, and can successfully treat cancers such as testicular carcinoma and various childhood leukaemias. However, they are not effective for all types of cancer.

Importantly, these chemotherapeutics can also give rise to secondary malignancies, as seen in particular following the initially successful treatment of childhood tumours[5] or testicular carcinomas[6] with chemotherapy. Additionally, they cause substantial toxicity as they affect normal cells as well as cancer cells; generally, they compromise not only the rapidly dividing cells of the haematopoietic system, the gut and hair follicles but also the function of post-mitotic tissues such as the heart muscle and peripheral nerves. This is one reason why these early chemotherapeutics have been considered 'dirty' drugs, although a precise definition and application of this term is difficult (BOX 1).

Timeline | **Selected key discoveries and approvals of anticancer drugs**



Anticancer drug development can be broadly divided into three waves. First wave (blue outline): chemotherapeutics targeting DNA integrity, DNA replication or cell division. Second wave (red outline): inhibitors of signalling molecules, such as cell membrane receptors or intracellular kinases. Third wave (green outline): inhibitors of multicomponent, co-oncogenic (instead of oncogenic) effector systems and machineries apart from those involved in DNA replication and mitosis. The years indicated in the timeline are in accordance with a historical overview on cancer chemotherapy from the American Association for Cancer Research (AACR) centennial series[218]. ADC, antibody–drug conjugate; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; FDA, US Food and Drug Administration; HDAC, histone deacetylase; HSP90, heat shock protein 90.

Second-wave anticancer drugs

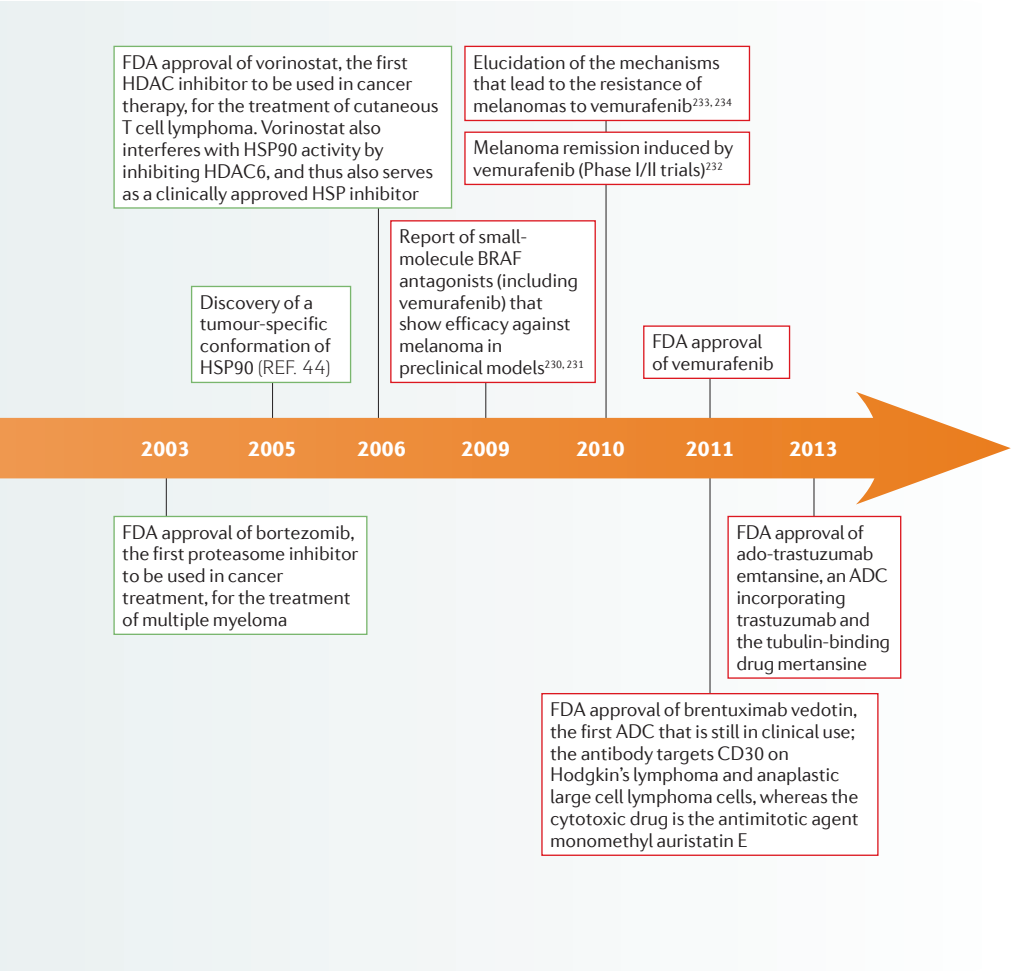
The limitations of the first-wave drugs that target DNA integrity and cell division, as well as advances in the understanding of the molecular drivers of cancer, fuelled efforts to develop anticancer drugs with a higher precision of molecular targeting, according to the century-old 'magic bullet' paradigm in which a disease-causing structure is directly targeted[7].

Some cellular targets are genetically altered in cancer cells and are essential to tumour development and survival (known as oncogene addiction). Other targets are not genetically altered but still more important in cancer cells than in normal cells (known as non-oncogene addiction)[8]. Oncoprotein targets, which are mainly involved in various signalling pathways, are primarily products of gene fusions, gain-of-function mutations or overexpressed oncogenes[8]. *MYC* was one of the first oncogenes identified that verified the concept of oncogene addiction: conditional knockout studies of *MYC* showed that established tumours shrank in response to the removal of this gene[9]. However, *MYC* is currently not amenable to direct pharmacological inhibition.

Nevertheless, over the past 15 years, several therapies targeting specific oncogenic signalling intermediates have been approved and have led to a revolution in the treatment of cancer (FIG. 1 (TIMELINE); TABLE 1). The specificity of these targets for cancer cells provided an opportunity to develop so-called 'clean' or 'smart' drugs (see BOX 1 for caveats associated with the use of these terms). The pioneering example is imatinib (Gleevec; Novartis), a small-molecule inhibitor of BCR–ABL kinase, which was approved in 2001 for the treatment of Philadelphia chromosome-positive (Ph^+) chronic myeloid leukaemia (CML)[10]. Other key signalling pathways or oncogenes that have been targeted with small molecules include members of the epidermal growth factor receptor (EGFR) family; the RAS–RAF–MEK (MAPK/ERK kinase)–ERK (extracellular signal-regulated kinase) pathway; the Hedgehog signalling pathway; and the JAK (Janus kinase)–STAT (signal transducer and activator of transcription) pathway (TABLE 1).

Another group of second-wave drugs comprises monoclonal antibodies targeting cell-surface receptors that are expressed preferentially on cancer cells compared to normal cells (BOX 2). The pioneer in this class is trastuzumab (Herceptin; Genentech/Roche), a monoclonal antibody that is specific for the receptor tyrosine kinase HER2 (also known as ERBB2 and Neu), which has proved to be successful in the treatment of the subset of ~25% of patients with breast cancer in whom HER2 is overexpressed[11]. ADCs could also be considered to represent an important subset of second-wave anticancer drugs, bringing together some of the advantages of first-wave drugs with the greater specificity of second-wave drugs (BOX 2).

Some signalling intermediates also represent examples of non-oncogene addiction in cancer cells; that is, they are not necessarily activated by tumorigenesis but they become essential for cells that undergo malignant transformation[8]. Examples (also previously



classified as non-oncogenes with addiction potential)[8] include checkpoint kinase 1 (CHK1), which is essential for the avoidance of extensive replicative stress[12], and mammalian target of rapamycin (mTOR), a kinase that controls the extent of overall protein synthesis[13].

In general, the side effects of agents that target signalling intermediates, and in particular oncogene products, are relatively milder than those of agents that directly target the DNA structure or the mitotic spindle apparatus. This is expected, as the targets of these agents are unique to — or at least largely confined to — cancer cells. However, after over 10 years of clinical experience, the development and application of such targeted drugs is facing challenges. Primarily, acquired resistance often leads to relapse, and in some tumour types such relapses represent the rule rather than the exception.

Mechanisms of resistance to drugs that target signalling intermediates. For many small-molecule inhibitors of signalling intermediates, various resistance mechanisms have been described (for a discussion of resistance mechanisms against tyrosine kinase inhibitors, see REFS 14,305). For imatinib, such mechanisms include the emergence of mutations in BCR–ABL that preclude the binding of imatinib, as well as the amplification of BCR–ABL or the overexpression of multidrug-resistance protein 1 (MDR1; also known as P-glycoprotein 1)[15], leading to treatment failure. As demonstrated in the IRIS trial[16], 7% of patients with chronic-phase CML progressed to the accelerated phase or blast crisis within 5 years of treatment, and 30% of patients with newly diagnosed Ph^+ CML did not achieve a complete cytogenetic response within 1 year of receiving imatinib treatment.

To overcome this resistance, additional inhibitors were developed that targeted alternative binding sites to the one targeted by imatinib (such as an SRC homology 2 (SH2) kinase domain inhibitor)[17] or that altered the conformational control of BCR–ABL[18]. Combinations of allosteric inhibitors with ATP-binding site inhibitors were also evaluated[19]. Although these new inhibitors overcame, to some degree, the acquisition of resistance due to changes in BCR–ABL, it turned out that cancer cells can bypass BCR–ABL inhibition by activating alternative signalling pathways for survival, such as the WNT–NFAT (nuclear factor of activated T cells)[20] or TGFβ–FOXO (forkhead box O)[21] pathways. Such mechanisms of resistance — that is, mutations that render a drug unable to bind to its target, or the activation of compensatory pathways for survival — have been observed for many other small-molecule anticancer drugs (TABLE 1).

Monoclonal antibodies are also not immune to the development of resistance or waning of efficacy[22]. For example, the development of trastuzumab-specific antibodies often leads to a failure to induce long-term remissions. Moreover, the activation of alternative signalling pathways can affect the efficacy of monoclonal antibodies. For example, acquired resistance to trastuzumab occurs through the activation of the phosphoinositide 3-kinase (PI3K)–AKT signalling pathway[23]. Mechanisms that prevent trastuzumab–HER2 interactions (such as epitope masking by mucin 4) have also been described[24,25]. Mechanisms of resistance for other monoclonal antibodies are summarized in TABLE 1.

Overall assessment of drugs targeting signalling intermediates. When based on molecular biomarkers and carefully tailoring to specific patient groups, the targeting of single oncoproteins has been a highly successful strategy in treating cancer. However, the development of resistance to these agents has highlighted how adaptive and heterogeneous tumours are.

One way to overcome this plasticity of tumours may be through the use of molecules that simultaneously inhibit multiple signalling intermediates — an approach that is sometimes referred to as 'polypharmacology'[26,27]. Indeed, over the past few years there have been several regulatory approvals for such compounds; for example, sunitinib (Sutent; Pfizer), sorafenib (Nexavar; Bayer/Onyx) and pazopanib (Votrient; GlaxoSmithKline) all target multiple tyrosine kinases and they have been successfully used to treat renal cell carcinoma and other tumours[28] (TABLE 1).

Another strategy is based on combining drugs that target different oncogenic signalling pathways, which could overcome the development of resistance due to the activation of alternative signalling pathways in cancer cells. One example is the combination of the BRAF inhibitor dabrafenib (Tafinlar;

Box 1 | **'Dirty' versus 'clean' drugs — the pitfalls of simplistic designations**

Drugs that target DNA or multicomponent machineries of cancer cells are sometimes referred to as 'dirty' drugs, whereas inhibitors of signalling intermediates are considered 'clean' drugs. This simplistic terminology is problematic, as the term 'dirty' in this context can have various different connotations, as discussed below.

Target molecule specificity. 'Dirtiness' increases with the number of different molecular species that a drug binds to in a cell. A drug often targets several structurally related proteins, such as kinases or histone deacetylases (HDACs), and this situation is sometimes referred to as polypharmacology[26,27].

Number of components in a complex of target molecules. When a drug targets a generic cellular complex composed of several proteins — for example, the DNA replication machinery or protein folding apparatus — it is expected to have pleiotropic effects.

Target molecule function. Some target molecules are nodes and govern a multitude of downstream pathways. For instance, this is true for regulators of transcription and chromatin structure. Again, the expected effects are pleiotropic.

Cancer cell specificity. The ultimate goal of a developing a clean drug is to only target cancer cells, leaving normal cells completely untouched. Dirty drugs are expected to be harmful to normal cells as well. The three criteria mentioned above seem to represent the mechanistic basis for this.

Since the discovery of oncogenic signalling pathways (and even before it), it seemed desirable to develop clean drugs that target only cancer cells by interacting with a single cancer-specific target molecule, governing a single downstream pathway. This concept is challenged by two major questions.

First, can clean drugs actually be identified? In many cases, it has turned out that even signalling inhibitors developed with a single molecular target in mind have several targets. For example, imatinib targets not only BCR–ABL but also KIT and platelet-derived growth factor receptor (PDGFR). It is envisioned that most — if not all — putatively single-target drugs actually affect the function of additional molecular targets, and this may be important to their efficacy. In the case of kinases, proteomics approaches such as the use of Kinobead technologies revealed that many rationally designed kinase inhibitors target more than one kinase owing to the conserved nature of the ATP-binding pocket[152]. Moreover, some oncogenic signalling intermediates gain their roles by overexpression, not mutation — for example, HER2 in breast carcinoma and epidermal growth factor receptor (EGFR) in colon carcinoma. Thus, at least some targets are also expressed in normal cells, resulting in an additional source of dirtiness.

Second, are clean drugs desirable? As outlined in this article, the clinical benefit of a drug that specifically targets signalling intermediates and thus comes relatively close to the ideal of a clean drug has so far been limited. Despite their successes in some haematopoietic malignancies, the development of resistance towards such 'magic bullets' remains a major problem in solid tumours. A broader range of molecular targets and/or a broader effect of such targets (termed dirtiness by the above criteria) may mean that the anticancer effects of a drug are greater and more sustainable, despite the increased likelihood of unwanted side effects.

Overall, clean drugs seem to be more of an ideal than a reality. Moreover, narrowly focusing on this goal may not necessarily turn out to be more productive.

GlaxoSmithKline) with the MEK inhibitor trametinib (Mekinist; GlaxoSmithKline) to treat *BRAF*^{V600E}-positive melanoma. In a Phase I/II trial, this combination had higher efficacy than the BRAF inhibitor alone, with a median progression-free survival time of 9.4 months with the combination versus 5.8 months with dabrafenib alone[29].

Other examples include combining erlotinib (Tarceva; Genentech/Roche), an EGFR inhibitor, with the multikinase inhibitor sorafenib[30] or the MET inhibitor tivantinib[31] to treat non-small-cell lung cancer (a progression-free survival of 3.4 months was observed for the combination versus 1.9 months with erlotinib alone in the first study; and a progression-free survival of 3.8 months with the combination versus 2.3 months with erlotinib alone was observed in the second study). At present, however, it is too early to tell whether such combinations will ultimately yield more sustainable results.

Overall, these observations make it clear that — with the exception of certain haematopoietic malignancies such as Ph$^+$ CML — targeting mutant or overexpressed oncoproteins is unlikely to represent a stand-alone basis for long-term successful cancer therapy, particularly for solid tumours. Most solid tumours carry a much larger spectrum of gene mutations than most haematopoietic malignancies (as an exception, however, non-Hodgkin's lymphomas have similar numbers of mutations to many solid tumours)[32]. This seems to enhance the flexibility of solid tumours to activate alternative oncogenic signalling routes when one is inhibited. Consequently, in addition to signalling inhibition, targeting entirely different cellular machineries may provide an opportunity for substantial progress in anticancer therapy.

Third-wave anticancer drugs

Given the limitations described above of drugs that target signalling intermediates, it seems worthwhile to revisit the principles that underlie the successes of classical chemotherapeutics. Rather than targeting single molecules, these chemotherapeutics target three broad cellular machineries: DNA replication, DNA repair and cell division. These machineries are required for cancer cell proliferation and survival. Is it possible to expand on this group to include other cellular machineries and effector systems that are essential for cancer cells, and that are more important for cancer cells than for normal cells?

In the past 10 years, several further such machineries have been characterized to understand their role in cancer and the putative antitumour effects that occur when they are inhibited. It has turned out that, similar to DNA replication, these machineries are required for tumour cell proliferation and survival with considerable selectivity (FIG. 2). This phenomenon is explained by the existence of chronic stress conditions in tumours and the ensuing non-oncogene addiction of cancer cells; these have been recognized as novel hallmarks of cancer[8,33,34] (TABLE 2). Targets that fall under this category and that have been the subject of most investigation are discussed in more detail below. In addition, newly emerging targets of this kind are highlighted in FIG. 2 and BOX 3.

Targeting protein folding and proteotoxic stress. Among the chronic stress conditions experienced by tumours, permanent proteotoxic stress is predominant. This induces a cancer cell-specific heat shock response[35] and leads to a strong dependence on heat shock proteins (HSPs) for proper refolding of stress-misfolded proteins to prevent conformational aberrations and aggregation.

Why is this kind of stress much more common in tumours? Various genetic anomalies that frequently occur in cancer cells contribute, including aneuploidy, copy number variation (CNV), microsatellite instability (MSI) in coding regions, missense and other mutations and/or aberrant overexpression of oncogenes[36]. These genetic anomalies lead to

Table 1 | **Selected pioneering anticancer drugs that target signalling intermediates and cell-surface molecules***

Drug	Target (or targets)	Indications	Mechanisms of resistance	Refs
Small molecules				
Imatinib (Gleevec; Novartis)	BCR–ABL fusion protein, resulting from a chromosomal translocation (Ph chromosome)	Ph$^+$ CML	Mutations in BCR–ABL; activation of alternative pathways (WNT–NFAT, TGFβ–FOXO)	10,239
Lapatinib (Tykerb; GlaxoSmithKline)	HER2 and EGFR	Metastatic breast cancer	Activation of PI3K–AKT signalling independently of HER2	240,241,242
Vemurafenib (Zelboraf; Roche/Plexxikon)	BRAF	Melanoma	Alternative activation of MEK–ERK signalling	29, 230–234, 243–246
Vismodegib (Erivedge; Curis/Genentech)	SMO (regulator of sonic hedgehog signalling)	Basal cell carcinoma, medulloblastoma	Loss of PTCH, amplification of GLI2, mutation of SMO	247–249
Ruxolitinib (Jakafi; Incyte/Novartis)	JAK1 and JAK2	Myelofibrosis	Kinase domain mutations	250–252
Gefitinib (Iressa; AstraZeneca)	EGFR	Non-small-cell lung cancer with EGFR mutation	MET amplification (and consecutive microRNA expression) and secondary EGFR mutation	253–256
Sunitinib (Sutent; Pfizer)	Multiple tyrosine kinases, including VEGFRs, KIT and PDGFRs	GIST, renal cell carcinoma	Kinase mutations, and many others	257,258
Sorafenib (Nexavar; Bayer/Onyx)	Multiple tyrosine kinases, including VEGFRs, KIT and PDGFRs	Hepatocellular carcinoma, renal cell carcinoma	Activation of PI3K–AKT signalling	259,260
Pazopanib (Votrient; GlaxoSmithKline)	Multiple tyrosine kinases, including VEGFRs, KIT and PDGFRs	Renal cell carcinoma, soft tissue sarcoma	Not yet characterized, clinically comparable to sunitinib	261
Tivantinib‡	MET and HGFR, but also MET-independent cytotoxicity	Hepatocellular carcinoma	Not yet characterized	262
Monoclonal antibodies				
Trastuzumab (Herceptin; Genentech/Roche)	HER2	HER2-positive breast cancer	Induced PI3K activation; epitope masking	11,23–25, 241,263,264
Brentuximab vedotin (Adcetris; Seattle Genetics)	CD30	Hodgkin's lymphoma, anaplastic large T cell lymphoma	Limited efficacy to begin with; improved by drug conjugation (brentuximab vedotin; see BOX 2)	265
Rituximab (Rituxan/ Mabthera; Biogen Idec/ Genentech/Roche)	CD20	B cell lymphoma, especially follicular non-Hodgkin's lymphoma	Reduced CD20 expression	266
Cetuximab (Erbitux; Bristol-Myers Squibb/Eli Lilly)	EGFR	Colorectal cancer, head and neck cancer	Activation of KRAS, BRAF, PI3K, loss of PTEN and mutation of EGFR	267–269,304
Bevacizumab (Avastin; Genentech/Roche)	VEGF	Colorectal cancer	Complex resistance mechanisms	270
Ipilimumab (Yervoy; Bristol-Myers Squibb)	CTLA4	Melanoma	Indoleamine 2,3-dioxygenase activity	157,159,271
Nivolumab‡	PD1	Melanoma, renal cell carcinoma	Indoleamine 2,3-dioxygenase activity	160,271

CML, chronic myeloid leukaemia; CTLA4, cytotoxic T lymphocyte antigen 4; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FOXO, forkhead box O; GIST, gastrointestinal stromal tumour; GLI2, GLI family zinc finger protein 2; HGFR, hepatocyte growth factor receptor (MET); JAK, Janus kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T cells; PD1, programmed cell death protein 1; PDGFR, platelet-derived growth factor receptor; Ph$^+$, Philadelphia chromosome-positive; PI3K, phosphoinositide 3-kinase; PTCH, Patched homolog; PTEN, phosphatase and tensin homolog; SMO, Smoothened; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. *For a thorough review on kinase inhibitors, see REF. 272. ‡Not yet approved by regulatory agencies.

quantitative imbalances in the stoichiometry of components within protein complexes, and to the synthesis of proteins with aberrant primary structure. This dramatically enhances the need for chaperone-supported protein folding to prevent the aggregation of growth signalling mediators and survival factors — for example, HER2, BCR–ABL, AKT and anaplastic lymphoma kinase (ALK)[36]. The higher level of reactive oxygen species (ROS) in tumours further contributes to proteotoxic stress by protein oxidation[37], as does the acidotic and hypoxic environment[36]. Consequently, the accumulation of misfolded and non-complexed proteins in cancer cells creates a high demand for HSPs, in particular for HSP90 (REF. 38).

HSP90 is part of a multicomponent complex — which includes HSP70, co-chaperones and the E3 ubiquitin ligase CHIP (carboxy terminus of HSP70-interacting protein) — and is essential in guiding the refolding of stress-misfolded polypeptide 'clients' into mature functional proteins. Intimately linked to this support function, the HSP90 complex also regulates levels of the client proteins[35,38]. In many tumours, the expression of HSP90 is upregulated to support malignant transformation — for instance, by promoting the stabilization of the oncogenic mutant p53 protein[39–41] or the anti-apoptotic signalling kinase AKT[42].

Box 2 | **Anticancer antibodies**

Molecules on the surface of cancer cells can be targeted by therapeutic antibodies[153]. Following on from the pioneering example of HER2-specific monoclonal antibodies for the treatment of HER2-positive breast cancer, several other antibodies that target cell-surface receptors have been approved, and many others are currently under evaluation. However, tumour heterogeneity with regard to antigen expression represents a major reason for their therapeutic failure[154] (TABLE 1).

As an extension from using antibodies alone, they have been conjugated to cytotoxic drugs — an approach that has taken more than two decades to demonstrate its potential, but is now one of the most active areas in anticancer drug development. Two examples of antibody–drug conjugates (ADCs) are the recently approved ado-trastuzumab emtansine (Kadcycla; Genentech/Roche) and brentuximab vedotin (Adcetris; Seattle Genetics). Ado-trastuzumab emtansine, a conjugate of a HER2-specific antibody and a microtubule inhibitor, provided clinical benefit for patients who had previously shown progression with trastuzumab treatment[155]. Brentuximab vedotin, a conjugate of a CD30-specific antibody and another anti-tubulin agent, showed promising results in Phase II trials for the treatment of patients with Hodgkin's disease[156] and also patients with anaplastic large cell lymphoma. This approach combines the broad activity of first-wave chemotherapeutics with an enhanced specificity for tumour cells versus normal cells, conferred by second-wave drugs that target signalling intermediates. Immune responses triggered by the antibody entity may further enhance efficacy.

Nevertheless, resistance mechanisms may arise that occur for both types of drugs — for example, through truncation or disappearance of the target epitope on the tumour, or through general adaptation of the cell to the impaired function of microtubules, as seen in taxol-resistant tumours. The clinical relevance of such resistance remains to be determined in future studies.

A more recent development involving the use of antibodies to treat cancer involves targeting immune receptors that would otherwise silence the cytotoxic T cell response. Such antibodies target cytotoxic T lymphocyte antigen 4 (CTLA4)[157] or programmed cell death protein 1 (PD1)[158] on T cells to overcome the silencing signal from dendritic cells or from tumour cells, respectively. CTLA4-specific antibodies were effective in treating patients with grade III or IV malignant melanoma, extending the median survival rate from 6 months to 10 months[159]. PD1-specific antibodies yielded a response rate of around 30% when they were used to treat patients with melanoma or renal cell carcinoma[160].

This dependence on the stress-induced (as opposed to constitutive) machinery of HSP complexes that comprise HSP90 (REFS 38,43) therefore creates an opportunity to target such HSP complexes for anticancer therapy. Moreover, as well as their enhanced expression, the structural properties of HSPs are altered in tumours, leading to differing affinities for their substrates. For example, HSP90 that is purified from tumours has a 100-fold stronger binding affinity for the HSP90 inhibitor 17-allylamino,17-demethoxygeldanamycin (17-AAG) than HSP90 from normal tissue[38,44], thus creating a cancer-specific therapeutic window. Numerous studies have demonstrated the anticancer effects of blocking HSP90; small-molecule inhibitors of HSP90 strongly interfered with the proliferation, survival and invasive or metastatic abilities of various tumour cell types, leading to the accumulation of ROS[45], perturbed chromosome segregation[46] and the degradation of oncogenic and anti-apoptotic proteins such as B cell lymphoma 6 (BCL-6)[47], macrophage migration inhibitory factor (MIF)[48] and mutant p53 (REFS 40,41) in cancer cells.

Several clinical trials of HSP90 inhibitors in solid cancers are currently underway or have recently been completed, and seem to be promising. For example, in a Phase II trial of 17-AAG plus trastuzumab versus trastuzumab alone, patients with HER2-positive metastatic breast cancer who had undergone progression with trastuzumab achieved a clinical benefit rate (complete response plus partial response plus stable disease) of 59% and a median progression-free survival of 6 months, with largely grade 1 side effects such as diarrhoea, fatigue, nausea and headaches[49]. A Phase III trial using the HSP90 inhibitor ganetespib (ganetespib plus docetaxel versus docetaxel alone) in patients with advanced non-small-cell lung carcinoma is currently underway (Galaxy 2; National Cancer Institute identifier: 9090–14; ClinicalTrials.gov identifier: NCT01798485). Ganetespib has received fast track designation from the FDA for second-line treatment of this tumour in combination with docetaxel (see the 12 September 2013 press release for further information).

Other HSPs are also being pursued, such as HSP70 (REF. 50), and the identification of a specific HSP110 mutant that sensitized colorectal cancer cells to chemotherapy (HSP110ΔE9)[51] provides another mechanism by which HSPs influence tumour growth and survival. With these encouraging developments, further work is required to not only inhibit HSPs but also accurately identify those patients who will best respond by developing predictive molecular biomarkers.

Targeting proteasome functions. Inhibiting the ubiquitin proteasome machinery is another approach for increasing proteotoxic stress in tumours. The ubiquitin proteasome machinery comprises a system of ligases that covalently link the polyubiquitin tag to proteins, leading to their proteolytic degradation in the proteasome[52–54]. So far, two proteasome inhibitors have been approved for treating multiple myeloma: bortezomib (a decade ago) and, more recently, carfilzomib (Kyprolis; Onyx) (TABLE 2).

Similar to HSPs, the enhanced demand for protein degradation and proteasome function in tumour cells may result from aneuploidy and the disproportionate synthesis of protein-complex members. Along this line, a proteasome component, proteasome 26S subunit ATPase 2 (PSMC2), was ranked highest among the recently identified CYCLOPS (copy number alterations yielding cancer liabilities owing to partial loss) gene products; that is, the partial genomic loss of *PSMC2* was frequent in cancer cells (10% in a panel of >3,000 cancers of various origins), and the suppression of PSMC2 by small interfering RNA (siRNA) led to strong suppression of proliferation in cancer cells that had partially lost the corresponding gene[55]. This underscores how strongly these cells depend on proteasome integrity, making it even more plausible that many cancer cell types are particularly susceptible to proteasome inhibition[55]. In addition, based on a synthetic lethality screen, the use of proteasome inhibitors was suggested as a strategy for eliminating cells with oncogenic RAS[56].

How can the ubiquitin proteasome machinery be targeted pharmacologically? The classical compounds such as bortezomib and carfilzomib bind to proteasomal components and inhibit their proteolytic activity. Bortezomib interacts with the catalytic 20S core subunit of the proteasome, masking a threonine residue that is essential for chymotrypsin-like proteolysis[57]; carfilzomib acts in a similar fashion[58]. Proteasome inhibitors were evaluated in pivotal clinical studies for the treatment of multiple myeloma. The APEX Phase III trial with bortezomib revealed an overall response rate of 43%[59], and the objective response rates to carfilzomib were 42–52%[60].

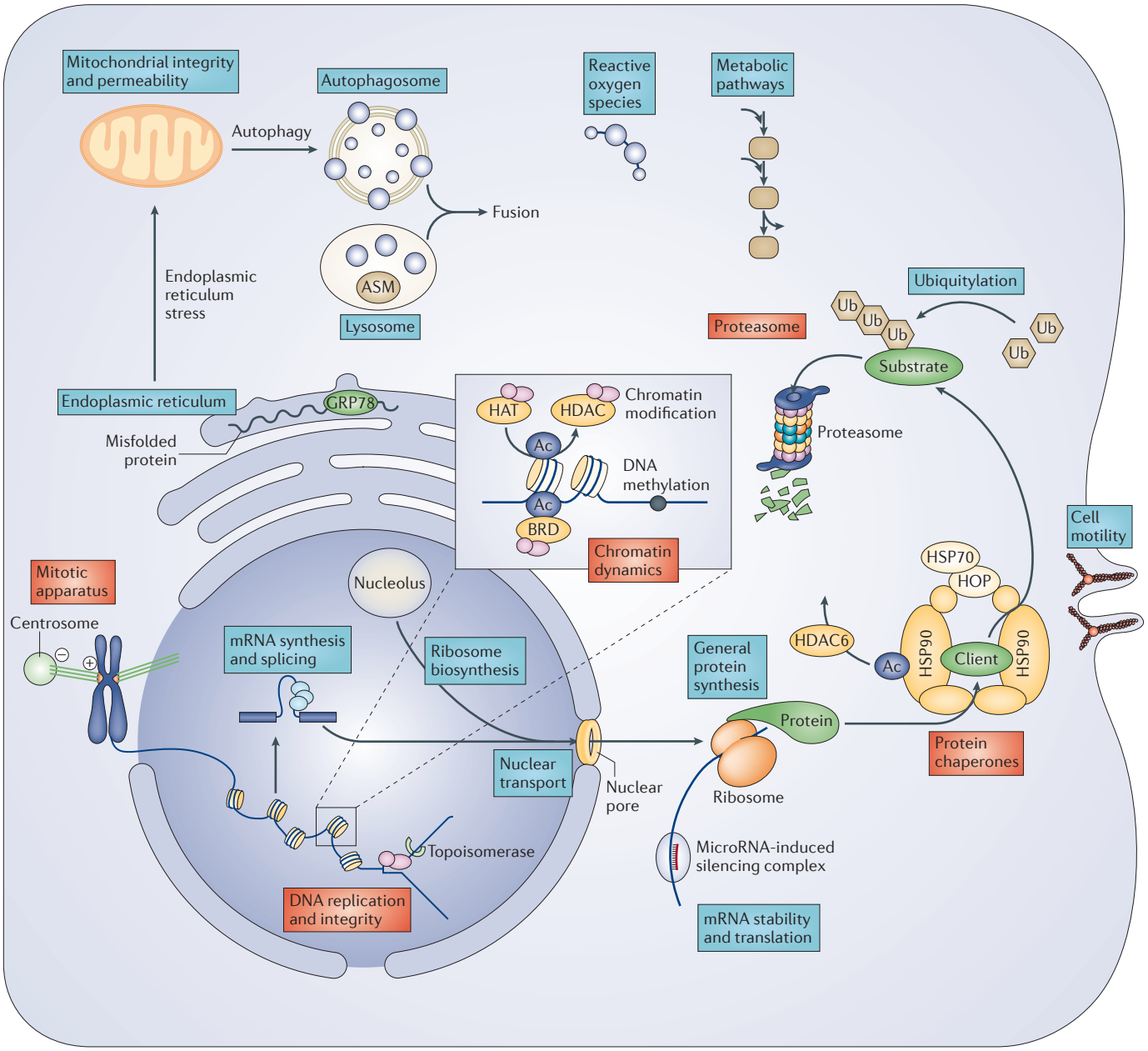


Figure 2 | **Cellular multicomponent machineries as current and future targets for anticancer drugs.** Current targets (shown in red boxes) include: DNA replication and integrity; the mitotic apparatus; chromatin; protein chaperones; and the protein degradation apparatus (the proteasome). Drugs that target DNA replication and integrity act via the following mechanisms: by crosslinking nucleobases in DNA and blocking DNA replication; by inhibiting DNA repair; by inserting planar polyaromatic molecules between DNA base pairs and stabilizing the DNA–intercalator–topoisomerase II ternary complex; by interfering with the polymerization of DNA (for example, via the incorporation of nucleoside analogues); and by inhibiting nucleotide synthesis, typically using antagonists of ribonucleotide reductases or thymidine synthetase. Drugs that target the mitotic apparatus act by binding to the inner portion of microtubules (the '–' end; for example, taxanes and epothilones), presumably leading to stabilization and enhanced rigidity of the spindle. Vinca alkaloids bind to the '+' end of microtubules — that is, the end that usually elongates the microtubule by adding subunits of α- and β-tubulin — thereby destabilizing the microtubule. Chromatin modification can be targeted by drugs that act on cellular enzyme complexes such as histone deacetylases (HDACs), bromodomain-containing proteins (BRDs) and DNA methyltransferases. Protein chaperones assist in refolding mutated or stress-misfolded proteins. Complexes consist of the heat shock proteins HSP90 and HSP70 (both of which are ATPases), as well as HSP90-organizing protein (HOP; also known as STIP1), multiple co-chaperones, adaptor proteins, the ubiquitin E3 ligase CHIP (carboxy terminus of HSP70-interacting protein) and the associated HDAC6 (a positive regulator and a cytoplasmic deacetylase that keeps HSP90 deacetylated and active). Drugs can inhibit HSP90, HSP70 or HDAC6. Drugs can inhibit different protease activities — for example, chymotrypsin-like activity, trypsin-like activity and/or caspase-like activity — within the 26S proteasome to disrupt the protein degradation apparatus. The ubiquitylation machinery and ubiquitin retrieval can also be manipulated by small molecules, providing additional opportunities for interfering with proteasomal degradation. Future targets for third-wave anticancer drugs are illustrated in blue boxes. For more details on the proposed mechanism of action of these future targets, see BOX 3. ASM, acid sphingomyelinase; GRP78, 78 kDa glucose-regulated protein.

Table 2 | **Types of constitutive stress in cancer cells* and selected drugs that exploit and/or enhance this condition**

Stress condition	Targets	Drugs‡
Well established for clinical use		
Replicative stress[273,274]	Enzymes for nucleotide synthesis	Nucleoside analogues: for example, 5-fluorouracil (5-FU) and gemcitabine
	DNA polymerases	Nucleoside analogues: for example, gemcitabine or cytosine-arabinoside (AraC)
	Topoisomerases	Topoisomerase inhibitors: for example, anthracyclines (doxorubicin, daunorubicin, epirubicin), camptothecin and irinotecan
Mitotic stress[8,275,276]	Microtubules, mitotic spindle	Taxanes (paclitaxel, docetaxel), epothilones (ixabepilone, patupilone, sagopilone) and vinca alkaloids (vincristine, vinblastine)
DNA damage[8,136]	DNA	Platinum compounds (cisplatin, carboplatin, oxaliplatin) and alkylating agents (cyclophosphamide, ifosfamide)
More recently developed drugs		
Altered chromatin dynamics[280]	HDAC enzymes, DNA methyltransferases	SAHA[281,282] (vorinostat)§, romidepsin‖[72], LBH589 (panobinostat)[283], azacytidine¶[284] and decitabine¶[285,286]
Proteotoxic stress: protein folding[8,38,44,287]	HSP90	Geldanamycin, 17-AAG[44], ganetespib[288], NVP-AUY922[289], valproic acid[290,291] and HDAC6 inhibitors (for example, vorinostat)
Proteotoxic stress: protein degradation[8,52]	20S core unit of the proteasome	Bortezomib[102#] and carfilzomib[58,60,292,293]**
Drugs at investigational stage and recently discovered activities of established drugs		
DNA damage[8,136]	PARP enzymes	Olaparib[277], rucaparib[278] and veliparib[279]
Nucleolar stress[162,167,294]	RNA polymerase I, exosome, additional components of ribosomes and nucleoli	Actinomycin D and 5-fluorouracil, two established drugs, exert part of their activities by interfering with ribosomal RNA synthesis in the nucleolus
Transcriptional stress (demand for RNA synthesis)[295]	RNA polymerase I (rRNA), RNA polymerase II (mRNA, miRNAs) and RNA polymerase III (tRNA)	Actinomycin D and 5- fluorouracil, two established drugs, exert part of their activity by interfering with RNA synthesis
Altered requirements for RNA splicing[96]	Spliceosome components	Spliceostatin A
Ribosomal stress (demand for protein synthesis)[177,296]	Translational initiation factors, ribosomes	Translation inhibitors, currently used for cell selection *in vitro*; interferons and other activators of translational shutdown
Enhanced demand for protein transport (logistic stress)[186]	Transport machineries into and out of the nucleus and organelles, including mitochondria, autophagosomes and the secretory pathway	Inhibitors of nuclear export are available (for example, leptomycin B and derivatives)
ER stress[297,298]	Endoplasmic reticulum; SERCA	Thapsigargin; however, besides inhibiting SERCA[299], the drug also blocks fusion of the autophagosome with the lysosome[300], and it was reported to promote carcinogenesis[301]
Lysosomal membrane vulnerability[211,212]	Lysosome-located acid sphingomyelinase (ASM)	Cationic amphiphilic drugs (CADs): an array of drugs that are already in clinical use, albeit not for treating cancer. Cytotoxic CADs include terfenadine, siramesine, amlodipine and nortriptyline
Metabolic stress[8,302]	Metabolic enzymes	Methotrexate and aminopterin, two established drugs, antagonize dihydrofolate reductase; other drugs in development
Oxidative stress[8,303]	Producers and scavengers of reactive oxygen species	Piperlongumine

17-AAG, 17-allylamino,17-demethoxygeldanamycin; ER, endoplasmic reticulum; HDAC, histone deacetylase; HSP90, heat shock protein 90; miRNA, microRNA; PARP, poly(ADP-ribose) polymerase; rRNA, ribosomal RNA; SAHA, suberoylanilide hydroxamic acid; SERCA; sarcoplasmic/endoplasmic reticulum calcium ATPase. *Some of these types of stress were suggested to represent novel hallmarks of cancer[8]. ‡In the case of more recently developed drugs, references were added mostly to describe their clinical use. §Vorinostat was approved by the US Food and Drug Administration (FDA) in 2006 for the treatment of cutaneous T cell lymphoma. ‖Romidepsin was approved by the FDA in 2009 for the treatment of cutaneous T cell lymphoma. ¶Azacytidine was approved by the FDA in 2004 for treating myelodysplastic syndromes; decitabine was approved by the FDA in 2006 for the treatment of myelodysplastic syndromes. #Bortezomib was approved by the FDA in 2003 for treating multiple myeloma. **Carfilzomib was approved by the FDA in 2012 for treating multiple myeloma.

Other strategies that are being pursued to inhibit the ubiquitin proteasome machinery include interfering with ubiquitin removal from substrates upon entering the 26S proteasome[61] and inhibiting the de-ubiquitylating enzyme ubiquitin-specific peptidase 14 (USP14), which blocks ubiquitin retrieval before protein degradation[62]. In addition, other approaches being pursued include allosterically inhibiting a ubiquitin-conjugating E2 enzyme (cell division cycle protein 34 (CDC34)), which induces broad-range accumulation of otherwise ubiquitylated targets[63], or blocking neddylation. Neddylation is a ubiquitin-like protein modification that is required for the function of the cullin–RING domain of a subset of ubiquitin ligases; hence, diminished neddylation results in broadly

compromised ubiquitylation[64]. Interestingly, inhibition of USP7 was able to overcome bortezomib resistance in multiple myeloma[65], which indicates the broad range of effects that can be elicited by inhibiting this system.

Targeting chromatin modifications. Transcription and DNA replication are regulated by covalent chromatin modifications such as DNA methylation and numerous histone modifications including acetylation, methylation, ubiquitylation, sumoylation and phosphorylation.

DNA methylation is targeted by 5-azacytidine (a nucleoside analogue of cytidine) and 5-aza-2′-deoxycytidine (decitabine; an analogue of the natural nucleoside 2′-deoxycytidine); these two drugs were identified in the early years of anticancer drug development, but their epigenetic activities have only more recently been appreciated[66]. 5-azacytidine and decitabine were approved by the FDA for the treatment of myelodysplastic syndrome in 2004 and 2006, respectively. Their mechanism of action first requires incorporation of the nucleoside analogue into newly synthesized DNA, which is followed by the binding of DNA methyltransferases. Rather than mediating methylation, however, the bound enzymes lose their stability and undergo degradation[66]. When treated with 5-azacytidine in a randomized trial, patients with myelodysplastic syndrome had an overall response rate of 15.7% versus 0% with best supportive care[67]. A Phase III trial with decitabine showed a 17% overall response rate versus 0% with best supportive care[68].

The first class of drug candidates to specifically affect histone modification are the histone deacetylase (HDAC) inhibitors (TABLE 2)[69,70]. Two drugs in this class — vorinostat (also known as suberoylanilide hydroxamic acid (Zolinza; Merck)) and romidepsin (Istodax; Celgene) — are approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL). The pivotal study for vorinostat was a single-arm Phase II study in patients with CTCL in whom two conventional therapies had failed; when these patients were treated with vorinostat, the objective response rate was 30%[71]. In a similar study using romidepsin, the objective response rate was 25%[72].

As the inhibition of HDACs affects the expression of a broad range of genes, identifying the specific genes that mediate the anticancer effects of HDAC inhibition is challenging, but such studies are gradually making progress. For instance, in cells derived from testicular cancer, HDAC inhibitors induced the extraordinarily strong long terminal repeat promoter of an endogenous retrovirus, thereby driving the expression levels of the adjacent pro-apoptotic *TP63* gene, which is a p53 paralogue that leads to enhanced apoptosis[73].

Besides HDACs, bromodomain and extra-terminal (BET) proteins have recently received attention as targets for modulating chromatin dynamics[74–77]. One of these, bromodomain-containing protein 4 (BRD4), holds promise as a target in acute myeloid leukaemia (AML)[78]. BRD4 was identified in a short hairpin RNA (shRNA) screen from a collection of epigenetic regulators[78]. Its downregulation suppressed AML progression in an *in vivo* model, and the same outcome was achieved by a BRD4 inhibitor named JQ1 (REF. 78). The efficacy of JQ1 was partially explained by its ability to reduce MYC expression, thus representing a strategy (albeit an indirect one) for achieving the long-sought goal of pharmacologically targeting MYC[78].

Histone methyltransferases are also involved in the progression of many tumours[79] and represent promising anticancer drug targets. A prominent example is the histone methyltransferase enhancer of zeste homolog 2 (EZH2), which is the catalytic subunit of Polycomb repressive complex 2 (PRC2). EZH2 is a major target of the tumour-suppressive microRNA miR-101, and its expression is essential for cancer cell survival in various model systems[80]. Several small-molecule compounds have been developed to inhibit EZH2, and these have led to tumour regression in animal models of lymphoma[81–83] and rhabdomyosarcoma[84]. So far, these compounds have mostly been tested in tumours that carry an activating mutation of EZH2, thus resembling other drugs that target cancer-specific mutant oncoproteins (that is, second-wave drugs, as discussed above). However, EZH2 inhibition may have anticancer effects even in the absence of such mutations, which indicates that inhibitors of EZH2 may have a broader mechanism of action (that is, they may be considered as third-wave drugs). For instance, cancer cells can also become dependent on EZH2 by losing SWI/SNF complexes[85].

Notably, in the acute response to various anticancer agents, a subpopulation of cancer cells regularly escapes via a chromatin-mediated drug-tolerance mechanism. At least in a cell culture model, chromatin dynamics involving histone demethylation transiently induced a drug-tolerant state in a small tumour cell subpopulation, which could be selectively ablated by HDAC inhibitors[86], further raising the utility of drug candidates that target the complex machineries modifying histones and DNA.

Approaches and challenges for identifying new drug candidates that target cellular machineries. Several approaches can be taken to identify novel targets within cellular machineries that would be suitable for targeting with drugs, including siRNA-mediated knockdown of specific components or genetic tumour models. Once these targets have been identified, rational drug design can be used to develop small-molecule inhibitors.

Targeted siRNA- or shRNA-based knockdown screens of specific components of various cellular machineries can be used to identify components that are crucial to the function of the machinery and therefore to the tumour; examples include the identification of a proteasome subunit[55] or a nuclear export factor[87]. However, care must be taken to ensure high knockdown efficiency, as the components of cellular machineries are frequently stabilized by being part of a complex with a long biological half-life. Indeed, this longevity might be the reason why not all such targets seem to be essential for cancer cells in standard short-term siRNA screens.

Genetic tumour models can also enable screening strategies within an entire organism to identify suitable drug targets[88]. Strikingly, such an *in vivo* loss-of-function screen revealed a nuclear export pathway as the subject of oncogenic dysregulation[87], thus pointing to intracellular transport machineries as potential drug targets.

Once a cellular machinery has been identified — by knockdown or genetic ablation — as being essential for tumour growth, the next step involves the development of small molecules to inhibit it. The use of cell-based screens of small-molecule libraries, rather than assays for single enzymatic activities, is often most suitable when trying to identify drugs of this type. Of note, the screening assays need not be limited to cell death or survival as the only readout. Rather, it is expected that more specific reporter systems or immunodetection methods will reveal the activity of specific cellular machineries. The use of high-content fluorescence microscopy could aid such approaches[89].

In other cases, it is possible to carry out *in vitro* assays to determine the activity of a cellular machinery. Indeed, proteasome[90] and HSP90 (REF. 91) function have been rebuilt in a cell-free test tube system for more than a decade. This allows a more robust and direct assessment of how a putative drug acts biochemically; however, cell permeability and off-target effects remain to be determined.

Box 3 | **Expanding the spectrum of third-wave anticancer drugs**

In addition to the cellular machineries discussed in the main text, several other cellular machineries and effector systems may be potential targets for third-wave anticancer drugs.

Nucleolar architecture and ribosome assembly. Ribosomal RNA (rRNA) is synthesized and assembled in nucleoli. Virtually all DNA-damaging drugs interfere with rRNA synthesis owing to DNA damage. Actinomycin D, a DNA-intercalating drug that is still used to treat gestational trophoblastic neoplasia[161], inhibits RNA polymerase I (which is responsible for rRNA synthesis), and this induces selective death of cancer cells[162]. 5-fluorouracil (5-FU) is incorporated into rRNA and also inhibits exosomes[163], a machinery mediating RNA turnover. Following nucleolar stress[164], regulatory proteins — such as $p14^{ARF}$, ribosomal protein L11, nucleophosmin, and so on — are released from nucleoli[165–167]. In the future, compounds that interfere with ribosome assembly without damaging DNA may induce nucleolar stress and inhibit cancer growth without provoking secondary malignancies.

RNA splicing. Targeting splicing and spliceosomes may interfere with cancer progression[96]. Spliceostatin A interferes with spliceosome assembly[168,169], which led to cancer cell death in mouse models[170].

MicroRNA processing and activity. MicroRNAs (miRNAs) act as regulators of gene expression[171], and this includes many cancer-related genes[172]. Targeting single miRNAs — for example, via complementary nucleic acid-based agents — remains difficult *in vivo*. Nevertheless, the general machinery of miRNA synthesis and action may be amenable to targeting with small-molecule inhibitors[173]. Heat shock protein 90 (HSP90) inhibitors also interfere with the general miRNA factor Argonaute 2 (AGO2)[174]. However, downregulating the general production of miRNAs by reducing the expression of the Dicer enzyme led to increased tumour formation in mouse models of cancer[175]. Conversely, complete genetic loss of Dicer suppressed tumours[175]. Thus, at first glance the system appears to contain too many antagonistic components for practical exploitation. However, distinct pathways of miRNA maturation have been described in cancer cells, as reviewed recently in REF. 176, thus providing novel opportunities for interfering with these pathways.

General protein synthesis and ribosomal function may also represent drug targets in cancer[177]. Omacetaxine, a drug recently approved by the US Food and Drug Administration (FDA), may interfere with ribosome function[178] — for example, in imatinib-resistant chronic myeloid leukaemia (CML)[179].

Intracellular transport. The export machinery that takes macromolecules from the nucleus to the cytoplasm represents a druggable target. The eukaryotic translation initiation factor 4E (eIF4E) protein is active in mRNA export and translation, and acts in an oncogenic fashion[180]. Ribavirin, an antiviral drug, interferes with eIF4E function, perhaps by mimicking the mRNA cap structure and competing with the eIF4E–cap interaction[181]. Clinically, ribavirin showed activity against acute myeloid leukaemia[182], albeit in early studies that require broader validation. It was also active against breast cancer cells, correlating with eIF4E overexpression in luminal B breast cancer[183]. The nuclear export inhibitor leptomycin B, which targets exportin 1 (XPO1; also known as CRM1), was capable of inducing death in CML cells[184] and pancreatic cancer cells[185]. The translation of this finding into clinical applications is still pending, but seems to be more feasible with recently developed and less toxic EXP1 inhibitors[186,187]. Targeting transport between other cellular compartments remains to be evaluated for cancer therapy.

Metabolic pathways. Cancer cells engage metabolic pathways that are distinct from normal cells. Even under normoxia, cancer cells rely heavily on glycolysis for ATP generation rather than on the mitochondrial respiratory chain (known as the Warburg effect)[188]. The M2 isoform of pyruvate kinase (PKM2) is mostly active in fetal tissue, but also in many cancer cells, giving rise to enhanced glycolysis[189]. Tyrosine phosphorylation of PKM2 and its association partners enables the use of glucose metabolites for anabolic processes that are required for cell proliferation[190,191]. These metabolic perturbations seem to be amenable to manipulation by small molecules[192,193], although so far the Warburg effect has not been selectively exploited for clinical therapy. However, an example of the successful manipulation of cancer metabolism is with antifolate drugs, such as methotrexate and its precedent aminopterin, which block dihydrofolate reductase and thus broadly interfere with purine and pyrimidine synthesis — for example, in triple-negative breast cancer[194] and gestational neoplasms[161].

In some cases, tumour-associated mutations activate aberrant metabolic pathways, which can be viewed as second-wave drug targets. Isocitrate dehydrogenase missense mutations in glioma and leukaemia alter enzymatic activity and can be targeted by small compounds[195,196], raising high expectations for their translation into the clinic.

Metabolic enzymes do not necessarily represent single units but can be found in large machinery-like complexes, such as those containing glucokinase and the pro-apoptotic protein BAD[197], further increasing the opportunities for their therapeutic modulation. The availability of technologies to assess metabolomics may facilitate the future development of anticancer drugs that interfere with metabolic pathways in tumours.

The redox system. Cancer cells must endure substantial oxidative stress from reactive oxygen species (ROS). Piperlongumine enhances ROS levels to eliminate cancer cells[198], and its derivatives may thus be clinically useful[199]. Oxidative stress typically induces necrosis; furthermore, in addition to its important role in apoptosis, the tumour suppressor p53 was recently implicated in triggering necrotic cell death via direct cyclophilin D-mediated activity in the inner membrane of mitochondria[200].

Organelles governing cell survival. Cancer cells can be susceptible to substances that tip the balance between pro- and anti-apoptotic factors. For instance, drugs that mimic the BCL-2 homology 3 (BH3) domain of pro-apoptotic, mitochondrial B cell lymphoma (BCL) family proteins trigger the intrinsic pathway of apoptosis[201]. This is expected to increase mitochondrial priming[202] — that is, readiness to promote apoptosis. The prototype drug of this class, gossypol (or its *R*-(-) enantiomer AT-101), showed some encouraging outcomes in Phase II studies in non-small-cell lung cancer (NSCLC)[203] and in a high-risk group of patients with prostate cancer[204].

Related approaches include the manipulation of endoplasmic reticulum (ER) stress[205]. For instance, thapsigargin targets sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) and induces the death of prostate cancer cells[206].

Other efforts attempt to inhibit autophagy for cancer therapy[207,208]. However, this is a double-edged sword because autophagy can carry out both protective and destructive functions in the cell. Traditionally used to treat malaria, hydroxychloroquine inhibits autophagy by increasing the pH in the autophagosome[209]. A Phase I trial on its use for treating NSCLC was completed in 2011 (REF. 210).

Finally, targeting the integrity of lysosome membranes by blocking acid sphingomyelinase (ASM) through cationic amphiphilic drugs (CADs) leads to the release of cathepsin and cell death. This effect was observed to a far greater extent in cancer cells versus normal cells[211,212].

Cell motility. Cancer cell motility represents a prerequisite for invasion and metastasis. Taxanes have long been known to affect cell motility, beyond interfering with the polymerization of the mitotic spindle[213,214].

Scaffold proteins for actin networks — that is, Wiskott-Aldrich syndrome protein (WASP) and WASP family member (WAVE) proteins — can promote metastasis[215,216]. Also, WASF3, a client of HSP90, can be targeted indirectly by HSP90 inhibitors[217].

With regard to the composition of small-molecule libraries for screening, those containing natural products might increase the likelihood of finding a suitable lead compound. This is because natural products often have a more elaborate molecular shape that might be more well-suited to disrupting complex cellular machineries than simpler compounds typical of standard chemical libraries, and they may even have evolved to block such machineries (for example, to fight off competitors of their host organism)[92].

Disrupting cellular machineries may require the inhibition of macromolecular protein–protein interactions, as opposed to inhibiting enzymatic activities. Although this represents a pharmaceutical challenge, some interfacial inhibitors have been known for a long time (for example, vinca alkaloids acting on tubulin interactions). The principles of their action may serve as a model for developing new inhibitors of this type. For instance, such drugs bind at the interface where components of molecular machineries interact; their binding sites are typically generated by the movements of such machineries that temporarily open the interface and allow drug access; and the tight-fit requirements for this kind of binding typically allow only one stereoisomer of a compound to be active[93].

The molecular three-dimensional structure of a target machinery can also be a very helpful tool for identifying interactions and domains that are most amenable to binding small molecules. Since the first protein structures were determined by X-ray crystallography, considerable progress has been made, allowing the crystallization and structure determination of relatively large protein complexes or even of subunits that comprise several protein components. The use of cryo-electron microscopy and other approaches to examine macromolecular structures has further enhanced our options for determining the three-dimensional composition of even highly complex molecular machineries[94]. The resulting structural models provide opportunities for designing small molecules that target machineries that were previously anticipated to be non-druggable. Along this line, our structural understanding of the spliceosome was profoundly improved by combining the above-mentioned approaches[95]. Although this has not yet led to the *de novo* design of spliceosome inhibitors, it strongly facilitated the chemical modification of natural compounds that affect splicing, leading to compounds with greater efficacy and specificity[96].

Translating this large spectrum of approaches and compounds into clinically useful drugs will require expanded efforts to align molecular characteristics with the clinical phenotype. Recently reported strategies to obtain patient-based cancer therapeutics[97] and conduct co-clinical trials that concomitantly use animal models to validate cancer therapies[98] might accelerate this process.

Limiting factors and challenges for drugs that target cellular machineries. Many of the limitations of early chemotherapeutics are also anticipated for newer drugs that target cellular machineries. A lack of absolute specificity for cancer cells will almost inevitably lead to side effects, and predicting these based on the molecular mechanisms of action of the drugs is challenging. Empirically, inhibitors of HDACs and HSP90 were found to cause mostly manageable side effects of fatigue, nausea and diarrhoea[71,99]; in addition, 17-AAG causes liver toxicity[100,101]. Proteasome inhibitors can cause peripheral neuropathy and myelosuppression at a substantial rate, which led to the discontinuation of bortezomib therapy in 37% of the patients with multiple myeloma who were enrolled in the APEX study. Specifically, peripheral neuropathy and thrombocytopaenia were observed in 8% and 2% of patients, respectively[102]. Only further preclinical and clinical assessments will reveal whether these or additional toxicities can be tolerated and whether they are outweighed by the benefits of the treatment.

Recent research has revealed the chemoresistance of small, stem-cell-like cancer cell populations — for example, in glioblastoma[103] or transformed breast epithelia[104]. In the case of transformed breast epithelia, a large-scale drug screen showed the feasibility of specifically targeting such populations with small-molecule compounds[105]. It remains to be determined whether drugs that target cellular machineries are suitable for eliminating such small populations of cancer-initiating cells (sometimes referred to as cancer stem cells)[106].

As for any drug, the mechanisms by which the drug is metabolized need to be considered. This is exemplified by the HSP90 inhibitor 17-AAG, which is converted into a more bioactive compound by the enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1); the expression levels of NQO1 represented a major determinant of 17-AAG sensitivity in large panels of cancer cell lines[107,108].

Beyond tumour-cell-intrinsic mechanisms of chemoresistance, dense 'desmoplastic' tumour stroma can block the access of drugs to the tumour[109]. In the case of pancreatic cancer, for example, at least in animal models the barrier could be overcome by enzymatic digestion of the tumour stroma[110] or by blocking Hedgehog signalling[111], as paracrine Hedgehog signalling from neoplastic cells to stromal cells is required for the maintenance of stromal desmoplasia[112]. These studies were carried out with classical (first-wave) chemotherapeutics (for example, gemcitabine in this case), but it is anticipated that similar strategies will be required to overcome the stromal barrier for third-wave drugs targeting cellular machineries. Nevertheless, the tumour stroma itself also represents a target for established and prospective cancer drugs, providing new opportunities for the generation of anticancer drugs from all three waves discussed in this article[113].

Are there specific characteristics of cancer cells that determine their sensitivity? Optimizing the use of any type of anticancer drug requires strategies to identify tumours with a high likelihood of response — for example, using biomarkers[114]. Progress is being made in the identification of biomarkers for drugs that target molecular machineries; for example, in the case of HSP90 inhibitors, highly expressed MIF[48] and mutant p53 (REF. 40) in cancer cells render these cells more susceptible to the drug.

The identification and evaluation of biomarkers that predict drug efficacy can involve several strategies besides purely empirical marker detection in patient samples. These include screens to identify genes that influence the sensitivity of cells towards these drugs; the identification of downstream pathways that are modulated by these drugs; and assessing the impact of such pathways on tumour cell survival. Guidelines for developing such biomarkers are provided by a landmark article that links cancer-associated mutations with twelve signalling pathways[115]. However, at least in the case of colorectal cancer and the currently available therapeutic regimens, the search for robust biomarkers has yielded only a very small number of confirmed predictive biomarkers out of hundreds of candidates[116], pointing to the need for rigorous clinical evaluation of putative biomarkers — for example, according to REMARK guidelines[117].

Reasons for the tumour specificity of broadly active anticancer drugs. Despite the use of first-wave chemotherapeutics for more than half a century, it is still unclear why these drugs eliminate some types of cancer cells

while adversely affecting normal cells only to an extent that is tolerable. The same is true for the third wave of drug candidates (those that perturb cellular machineries) discussed above. However, general concepts for explaining the relatively higher sensitivity of cancer cells compared with normal cells are emerging. A landmark summary of such phenotypic hallmarks of cancer was provided more than a decade ago[33] and has recently been updated[34]. Another independent study expanded on the first six hallmarks of cancer, focusing on chronic cellular stresses of cancer cells that lead to their addiction to non-oncogene support systems[8].

One obvious feature of most cancer cells is their ability to proliferate without depending on external growth factors, without being inhibited by growth-suppressing factors, and without being limited by telomere shortening[33]. Cell proliferation requires DNA replication and mitosis, providing some (classical) explanation for the increased sensitivity of cancer cells to first-wave chemotherapeutics. However, some normal cells (for example, in the gut, haematopoietic system and hair follicles) proliferate at least as fast as — if not faster than — most cancer cells. Indeed, these cells are often most affected by chemotherapeutics, causing undesirable side effects. But how could some tumours still be eliminated without deleting these normal cells entirely? This question particularly arises with regard to cancer stem cells[106]. In many cancer types this tiny population of cells is believed to constitute the main reservoir for cancer cell regeneration, despite displaying a very slow division rate compared to the bulk of 'more differentiated' cancer cells.

So why should such slowly dividing cells still respond to first-wave anticancer drugs? Part of the answer may lie in the lack of checkpoints, which is a deficit associated with most — if not all — cancer cells as opposed to normal cells. These checkpoints respond to DNA damage or to chromosomal misalignment during mitosis. Under some circumstances, triggering the checkpoint causes cell death — a mechanism that helps to eliminate nascent cancer cells[118]. Sometimes, however, checkpoint activation only leads to temporary cell cycle arrest, thus protecting the cell from the deleterious consequences of trying to replicate or divide its chromosomes when it is damaged. In this scenario, a lack of checkpoint function, as found in cancer cells, removes this protective function and leads to enhanced sensitivity towards chemotherapeutics[119].

We propose that similar principles may apply to drugs that affect protein metabolism rather than DNA metabolism. Owing to their active proliferation and metabolism, and to frequent aneuploidy and copy number variation, cancer cells can be expected to synthesize, fold and degrade proteins at an enhanced pace. In addition, they may have weakened systems that would normally keep general protein synthesis in check. On the one hand, enhanced protein synthesis can be caused by mTOR–S6K (ribosomal protein S6 kinase) signalling, a pathway that is often constitutively switched on in cancer[120]. On the other hand, cells can regulate overall protein synthesis by PKR, a protein kinase that is also involved in the response to viral infection and interferon. PKR activity regulates cell proliferation[121], and PKR signalling was proposed to be compromised by RAS activity in cancer cells[122,123]. Thus, a lack of surveillance mechanisms that regulate protein synthesis, akin to a lack of protective cell cycle checkpoints, may constitute the basis for the enhanced sensitivity of cancer cells towards inhibitors of HSPs and protein turnover. This concept, however, awaits corroboration in future studies.

Comparison of the three waves of drugs

Anticipated advantages of drugs that target cellular machineries without directly affecting DNA integrity. Why could drugs that target protein turnover be more efficacious than the well-established chemotherapeutics that target DNA?

First, classical (first-wave) chemotherapeutics result in severe DNA damage and/or chromosomal instability, both in cancer cells and normal cells. Hence, if a patient survives the initial tumour, a long-term consequence of chemotherapy is a significant risk of developing secondary malignancies[5,6]. This scenario is less likely to occur with modulators of chromatin modification and protein turnover, as these compounds are not known to induce immediate DNA damage. However, it has to be noted that not all cancer-inducing compounds are also directly DNA-damaging (that is, mutagenic), as exemplified by the increased rate of breast cancer observed following postmenopausal hormone replacement therapy[124]. In addition, HSP90 inhibition can cause aneuploidy, at least in yeast[46], raising the possibility of some carcinogenic potential. Hence, as for any drug, the long-term toxicity of new compounds will need to be carefully assessed in large patient cohorts, which should include monitoring for cancer recurrence.

Second, drug candidates that target the machineries involved in protein folding or turnover have a broader range of biological effects than the classical inhibitors of DNA-associated machineries, which could provide more opportunities to hit the Achilles heel in a given tumour. Given the multitude of characteristics of cancer cells, we expect these new drug candidates to increase response rates. In the case of classical chemotherapeutics, the greatest successes have been achieved in lymphomas, childhood malignancies and testicular carcinoma. It is conceivable that the new wave of drugs targeting other molecular machineries might work best for a different spectrum of tumour entities, knowledge of which will emerge through clinical experience.

Anticipated advantages of drugs that target cellular machineries versus signalling intermediates. As discussed above, drugs that target signalling intermediates are prone to resistance development. If a signalling pathway is blocked by a drug, the tumour can activate an alternative pathway with overlapping downstream targets. In the case of a multicomponent cellular support machinery, such replacement seems less likely. For example, no other effectors in the cell can carry out the functions of the proteasome, HSPs or major chromatin modifiers.

But can we anticipate that resistance would not develop to broadly acting drugs? Cancer cells can overcome DNA damage caused by first-wave drugs by modulating damage signalling or by dampening death signals — for example, mutations in p53 are the best-studied alteration[125]. Consequently, it is expected that third-wave anticancer drugs that target cellular machineries will also give rise to resistance mechanisms. In the case of HDAC inhibitors, such resistance has been observed — for example, through the overexpression of HDAC1 (REF. 126) or through alterations in the downstream responses (such as autophagy and apoptosis)[127]. For the proteasome inhibitor bortezomib, resistance due to single-gene mutation and overexpression has been observed, altering proteasome subunit β-type 5 (PSMB5)[128].

Nevertheless, we propose that resistance to third-wave drugs is less likely to be absolute, as the targeted machineries are required for cancer cell survival. This is a key difference between second-wave anticancer drugs targeting signalling intermediates (for which tumours can activate alternative signalling pathways to become resistant) and third-wave anticancer drugs (for which this is not expected to occur). Both types of drugs,

Glossary

Antibody–drug conjugates
Therapeutics consisting of a tumour surface-targeting antibody (a whole monoclonal antibody or a single-chain variable fragment) linked via a chemical linker to a cytotoxic molecule with anticancer activity.

Biomarkers
Parameters that can be measured in patients — for example, to indicate the expression levels of a gene in tumour cells. In cancer treatment, biomarkers serve to indicate the likelihood of cancer progression, death, remission or a cure (these are known as prognostic biomarkers), or to assess how well a particular therapeutic regimen will work for a given patient (these are known as predictive biomarkers).

Hallmarks of cancer
The general phenotypic traits of cancers. The original article by Hanahan and Weinberg defines six such hallmarks: self-sufficiency in growth signals; insensitivity to anti-growth signals; evasion of apoptosis; limitless reproductive potential; sustained angiogenesis; and tissue invasion and metastasis. This list has since been expanded.

Kinobead technologies
The use of beads that are linked to kinase inhibitor molecules. These molecules bind to a subset of kinases from a cell lysate, allowing their subsequent identification and quantification by mass spectrometry.

Monoclonal antibodies
Antibodies generated by a clonal B cell population (traditionally fusion hybrids of individual B cells with a myeloma cell). For cancer therapy, monoclonal antibodies are used to target tumour-associated molecules on the surface of cancer and/or stromal cells or secreted factors.

Non-oncogene addiction
The phenomenon whereby cancer cells critically depend on a particular adaptive mechanism to a greater extent than normal cells, although the corresponding genes do not fulfil the criteria of oncogenes; this mechanism is typically composed of a complex multicomponent machinery.

Oncogene addiction
The phenomenon whereby cancer cells depend on the continuous hyperactivation of one or more oncogenes and their products for their survival.

Philadelphia chromosome
A reciprocal translocation of the chromosomes 9 and 22, designated t(9;22)(q34;q11), found in more than 90% of chronic myeloid leukaemias.

REMARK guidelines
Guidelines entitled 'Reporting recommendations for tumour marker prognostic studies', which recommend standards for the evaluation of, and reporting on, biomarkers in clinical studies on cancer, including "study design, preplanned hypotheses, patient and specimen characteristics, assay methods, and statistical analysis methods".

Small molecules
Organic compounds with a low molecular mass (the usual upper limit is 500–1,000 Da) that bind to specific macromolecules (such as proteins), thus altering their activity or function. Traditionally, small molecules with anticancer effects inhibit enzymatic activities, but more recently protein–protein interactions have also been successfully targeted.

Synthetic lethality
Originally defined as a combination of two genes that, when individually mutated, can be tolerated by an organism, but when mutated simultaneously, result in death. This concept was extended to cancer therapy; if a gene is synthetically lethal to a cancer-associated mutation of another gene, targeting (that is, inhibiting) the product of the first gene using drugs will also result in synthetic lethality.

Therapeutic window
The range of a drug dose that both confers the desired effect (such as a reduction in tumour growth) and can also be tolerated by the patient without causing prohibitively severe side effects as a result of damage to normal tissues.

however, can be limited by acquired resistance due to mutations in the target that affect drug binding or due to broadly active cellular adaptation mechanisms.

Anticipated synergies of broadly active drugs from different classes. Although largely unexplored, it is conceivable that combining more than one drug to target different complex cellular machinery components will increase the efficacy of cancer cell elimination. The following arguments are in favour of such combinations.

First, two drugs that work by different mechanisms often synergize to achieve efficient cell killing — for example, by inhibiting two pathways or processes when either alone may be sufficient for cell survival if not inhibited. This scenario is similar to the previously proposed principle of synthetic lethality in cancer treatment[129], although the original review elaborated more on how to exploit a specific cancer cell trait to achieve drug efficacy rather than emphasizing the combination of two cytotoxic drugs.

Second, even if they are present in a subset of cancer cells, resistance mechanisms are less likely to lead to a dominant resistant cell clone when two drugs targeting different cellular machineries are combined. This is comparable to the simultaneous treatment of bacterial infections with more than one antibiotic to avoid the outgrowth of resistant subclones[130].

Third, drugs that affect DNA integrity are already part of many clinical regimens that have proven to be of some benefit. When setting up clinical studies, combining an established drug with a new compound is often a more justifiable strategy than introducing an entirely new combination regimen.

Promising examples for drug combinations include combining chromatin modulators with conventional DNA-damaging chemotherapy, as the chromatin structure is increasingly recognized as being critical for the DNA damage response[131,132]. Similarly, interfering with proteasome activity was shown to restructure the chromatin profoundly, leading to a global decrease in histone H2B monoubiquitylation, which suggests a possible synergy between proteasome inhibitors and chromatin modulators[133]. Indeed, a proteasome transport factor, UV excision repair protein RAD23 homolog B (RAD23B; also known as HR23B), induced sensitivity towards HDAC inhibitors[134], providing a link between two cellular machineries that tumour cells depend on for survival. Finally, a cytosolic member of the HDAC family, HDAC6, turned out to be essential for HSP90 activity by keeping HSP90 deacetylated[135], and thus a HDAC6 inhibitor synergizes with 17-AAG to kill p53-mutant cancer cells[40].

Despite these promising scenarios, potential drug antagonism also needs to be taken into consideration. For instance, targeting the HSP90 complex leads to the destabilization of potent oncoproteins such as mutant p53, ERBB2 and MIF (which is a tumour promoter). However, simultaneously blocking the proteasome restores the levels of these proteins to some extent[40,41,48], potentially constituting undesirable negative interference of the two drugs.

Regardless, positive or negative interference of drugs that target cellular machineries constitutes a major, largely unexplored field of research that should lead to a more refined and efficient use of these drugs in cancer therapy.

Synergies of drugs that target signalling intermediates with drugs that interfere with cellular machineries. As discussed, genotoxic compounds still represent the vast majority of available anticancer drugs[136,137]. Progress has been achieved with the combination of classical first-wave chemotherapeutics with second-wave signalling inhibitors. Although either of the two alone frequently fails to be effective, combinations may yield more promising results. However, limited evidence is available for the clinical usefulness of such combinations, and most examples are still at the preclinical stage; examples that seem to show the most promise are discussed below.

One example of combining first- and second-wave drugs is provided by MEK inhibition to enhance the efficacy of neoadjuvant breast cancer chemotherapy, at least in xenografts[104]. The authors found that the levels of dual specificity protein phosphatase 4 (DUSP4), an ERK phosphatase, were low in fast-relapsing tumours. Correspondingly, inhibition of MEK synergized with docetaxel treatment in xenografts of basal-like breast cancer[104].

The activity of poly(ADP-ribose) polymerase (PARP) inhibitors can also be broadened by combining these drugs with kinase inhibitors[138–140]; PARP is an enzyme that is involved in single-strand DNA break repair and base excision repair. PARP inhibitors induce replication fork collapse following spontaneous single-strand breaks, which in turn triggers repair by homologous recombination (HR). These drugs were found to effectively kill breast cancer susceptibility 2 (BRCA2)- and BRCA1-mutated cancer cells via synthetic lethality by raising their need for HR, which is compromised in these cells[141]. However, resistance to PARP inhibitors has developed in some cases as a result of the emergence of revertants (via intragenic reversion of mutated BRCA2 back to functional BRCA2)[138]. Conversely, a new strategy has broadened the scope of PARP inhibitors by additionally interfering with cyclin-dependent kinase 1 (CDK1) activity[142], even in the presence of wild-type BRCAs.

Generally, inhibition of DNA repair mechanisms represents an attractive strategy for boosting the efficacy of genotoxic drugs. Each of the existing repair mechanisms is amenable to inhibition by small molecules[143], and PARP inhibition represents an example that has been the focus of much research so far. As many repair mechanisms are driven by complex protein machineries rather than by single enzymes, at least some of these candidates could arguably be considered as third-wave anticancer drugs.

Various chemotherapeutics act on their target cells particularly during DNA replication: that is, during the S phase of the cell cycle. This is exemplified by nucleoside analogues such as gemcitabine or cytosine-arabinoside (Ara-C). In this case, activation of the kinase CHK1 seems to protect the cancer cells from further damage, perhaps by inducing an intra-S checkpoint that allows the reconstitution of stalled replication forks at sites of DNA lesions. Thus, inhibition of CHK1 or of other kinases signalling to CHK1 seems to be a viable strategy for chemosensitization[119], especially when the effector kinase MAPK-activated protein kinase 2 (MAPKAPK2) is active to prevent translesion DNA synthesis[144].

Moreover, resistance to inhibitors of mitotic spindle dynamics, such as taxanes, epothilones or vinca alkaloids, can be overcome by signalling inhibitors. Some important determinants of resistance were identified by showing how a cancer cell responds to drugs that target the mitotic spindle[4,145,146]. Examples of resistance factors include activation of the Notch and Hedgehog signalling pathways, and their inhibition leads to the sensitization of prostate cancer cells to docetaxel, at least in animal models[147].

Along a similar line, the spindle assembly checkpoint (SAC) is triggered by a complex system of signalling intermediates. As a consequence, numerous factors govern this checkpoint, and targeting these factors can either block it, leading to severe chromosome missegregation, or permanently activate it, resulting in durable mitotic arrest and apoptosis. Examples include targeting the anaphase-promoting complex (APC; also known as cyclosome)[148] or CDC20 (REF. 149). Severely perturbed chromosome segregation, leading to apoptosis, can also be achieved by targeting the mitotic kinesin-like protein KIFC1 (also known as HSET), thereby compromising the function of another cellular machinery — the clustering of centrosomes[150]. Combinations of SAC-targeting drugs with drugs that damage the spindle microtubule seem to represent a particularly promising strategy for eliminating cancer cells[145,151].

Conclusions

The development of new anticancer drugs must not be limited to single targets involved in signal transduction; rather, it needs to include complex multicomponent support machineries that cancer cells are selectively addicted to for survival, such as chromatin modification, protein conformation and folding, as well as protein turnover, among others. Compounds targeting these potential Achilles heels may be less prone to circumvention by drug-induced single-target mutations or the activation of bypass pathways.

It is likely that these new compounds will not be used as monotherapies, and attaining their optimal clinical efficacy will require the molecular characterization of a given tumour to predict the drug class and drug combination with the highest likelihood of success. One of the key questions to be addressed in the future is whether the activity of specific oncogenic signalling pathways also determines the sensitivity of a cancer cell to inhibitors of HDACs, HSPs or the proteasome — that is, whether inhibitors of particular signalling intermediates could be used to sensitize tumours towards drugs that target cellular machineries and vice versa. In general, further research will be required to identify robust predictive biomarkers for the efficacy of these new drug candidates and to optimize synergies with existing regimens, which will hopefully allow them to drive the next leap forward in cancer treatment.

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Competing interests statement
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Synta announces fast track designation granted for ganetespib in non-small cell lung adenocarcinoma (12 September 2013; press release):
http://www.businesswire.com/news/home/20130912005700/en/Synta-Announces-Fast-Track-Designation-Granted-Ganetespib#.UsVcePRdVb4

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