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Review

Contemporary pre-clinical development of anticancer agents – What are the optimal preclinical models?

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ABSTRACT

The successful identification of novel effective anticancer drugs is largely dependent on the use of appropriate preclinical experimental models that should possibly mimic the complexity of different cancer diseases. The huge number of targets suitable for the design of new anticancer drugs is producing hundreds of novel molecules that require appropriate experimental models to investigate their mode of action and antitumour activity in order to select for clinical investigation the ones with higher chances of being clinically effective. However, our ability to predict the clinical efficacy of a new compound in the clinic based on preclinical data is still limited.

This paper overviews the *in vitro/in vivo* preclinical systems that are currently used to test either compounds with an unknown mechanism of action or compounds designed to hit cancer-specific or cancer-related molecular targets. Examples of experimental models successfully used to identify novel compounds are provided. Xenografts are still the most commonly used *in vivo* models in drug development due to their high degree of reproducibility and because, in some cases, particularly when orthotopically transplanted, they maintain several biological properties of the human tumours they derive from. Genetic models are very useful for target validation, but are often not sufficiently reproducible to be used for drug evaluation. The variety of animal models can be effectively used to optimally test drugs that presumably act by a defined mode of action, but final success is highly dependent on the ability of drug development teams to integrate different expertises such as biology, chemistry, pharmacology, toxicology and clinical oncology into a clever and well orchestrated plan that keeps in consideration both the complexity of cancer diseases, involving alterations of different pathways, and the complexity of drugs whose pharmacological properties are crucial to obtain the desired effects.

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1. Introduction

Over the last two decades, the empiric chemically-driven discovery of new anti-cancer agents has been replaced by biologically-driven anticancer drug discovery/development, mainly based on compounds acting on specific molecular targets. This is in fact the result of the improved biological knowledge of the molecular pathways found to be deregulated and/or over-activated in different tumours that has led to the identification of molecular targets (growth factor receptor, intracellular signalling molecules, etc.) against which drugs have been designed.[1–4] This increased biological knowledge has

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been associated with the development of high-throughput genomic and proteomic analyses, a high-throughput compound screening and structure-based drug design that allow the identification and development of a large number of molecules that specifically interfere with the above mentioned targets. Although the identification of new anti-tumour agents is mainly based on *in vitro* methodologies, the *in vivo* models are absolutely required to assess the pharmacological activity of a potential new drug in animal models in which the drug undergoes distribution in both neoplastic and normal tissues, is metabolised and eliminated. It should be considered that in a growing number of cases the activity of a new compound may depend not only on the intrinsic cellular sensitivity but also on other mechanisms related to the presence of the stroma, the hypoxic conditions, and neo-angiogenesis etc., all factors present *in vivo* but not *in vitro*.[5]

The typical development plan for a new anti-cancer agent involves sequential steps: *in vitro* assays, both cell based and molecular target-driven, for the identification of an active compound; *in vivo* studies to assess the potential antitumour activity; pharmacological studies to define drug absorption, distribution, metabolism, and elimination; and, finally, toxicological studies to define a safe starting dose in humans. All these structured steps, in particular the first ones, had to be adapted for the preclinical development of targeted agents (being the rationale at the basis of the new therapeutic

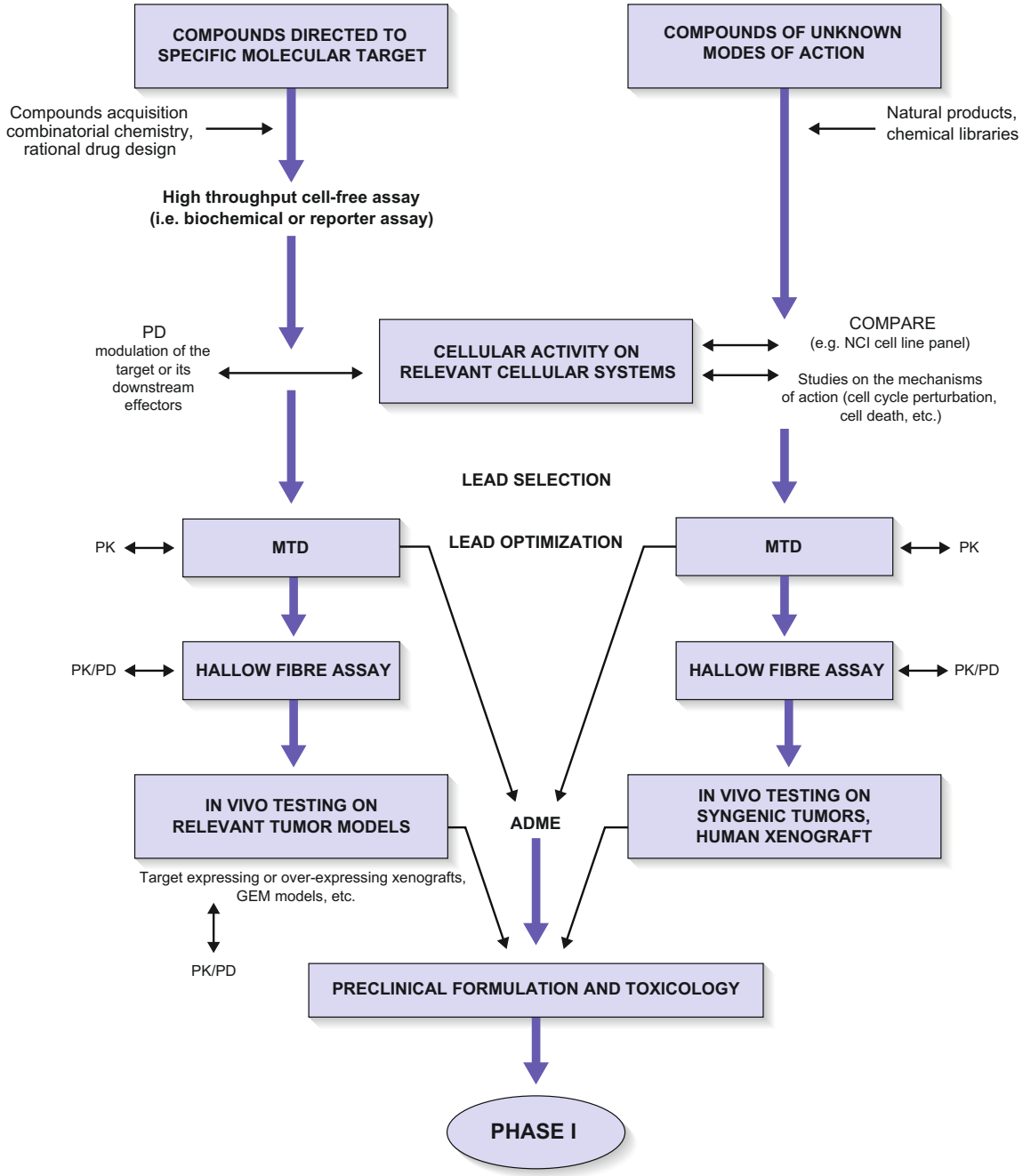

Fig. 1 – Preclinical development steps in the evaluation of new compounds.

strategy, different from the one for classical cytotoxic agents). We realise that the schematic division between classic cytotoxic agents and targeted drugs is often conceptually wrong and misleading, considering that most cytotoxic agents have indeed well defined targets, e.g. DNA or tubulin, and conversely most new agents are directed to targets that are not cancer specific, e.g. enzymes or factors expressed in both cancer and normal cells. Therefore, in this paper, we will overview the available preclinical experimental models to evaluate either compounds for which the target is non cancer specific or those that have been used for the development of cancer specific targets (Fig. 1). The description is associated to examples of drugs successfully developed by using these models.

2. Preclinical development of cytotoxic anticancer agents

2.1. *In vitro models*

2.1.1. *NCI screening model*

In 1990 the US National Cancer Institute (NCI) modified the screening system which was previously based on mouse tumour models.[6] It was realised that the efficiency of discovery of compounds clinically active against human solid tumours was very low, possibly due to major differences between rodent and human tumours.[7] The new screening system was based on cell lines derived from all the major human neoplasms that might mimic better the heterogeneity of the biological features of human tumours. In addition, the new programme made it possible to rapidly evaluate a large number of anticancer agents in a disease oriented manner. The initial panel of cell lines incorporated a total of 60 cell lines representing nine distinct tumour types (leukaemia, colon, lung, CSN, renal, melanoma, ovarian, breast and prostate cancer). To date, more than 85,000 compounds have been screened against this *in vitro* panel of short term assay. Compounds are tested over a 5-log concentration range against each of the 60 cell lines for their ability to inhibit the growth of, or to kill, the cells in a 2-day assay generating 60 dose-response curves. To facilitate the analysis and interpretation of the data, three end-points are calculated for each cell line: the GI_{50} value that is the negative $\log_{10}$ of the concentration required to inhibit the growth of that cell line by 50% (relative to untreated control cells), the TGI that is the negative $\log_{10}$ minimum concentration that causes total growth inhibition and the LC_{50} that reflects the negative $\log_{10}$ concentration needed to kill 50% of the cells. These data generate a characteristic fingerprint of cellular response, the 'Mean Graph', profiling the sensitivity/resistance of the compound on all the cell lines. Compounds with similar mechanisms of action tend to have similar patterns of growth inhibition in the 60 cell line screen and then similar Mean Graphs. A computerised pattern recognition algorithm was developed by Paull and colleagues[8], the COMPARE, to quantify the similarity of Mean Graphs from different compounds. Initial findings revealed that compounds with similar Mean Graph patterns often shared a same or related mechanism of action. In contrast to fingerprints with patterns of similarity to standard agents in the database, compounds have been detected that produced strikingly different fingerprints. These COMPARE negative compounds indicate a unique mechanism of action. In this regard, the example of the proteosome inhibitor Bortezomid can be cited. Bortezomid was submitted to the NCI screening in July 1995 together with a group of chemical analogues by Proscript Pharmaceuticals. The pattern generated in the screen was COMPARE negative, suggesting a mechanism of action different from the known anticancer agents. Testing in the hollow fibre model showed activity, confirmed in the subsequent xenograft model. Bortezomib was approved by the US Food and Drug Administration (FDA) in March 2003.[9]

From the early 1990s, a programme to characterise 'Molecular Targets' within the 60 cell line panel was initiated.[7,10] This led to the accumulation over the years of data on the mutational status of genes important for cancer (e.g. *p53* and *Ras* genes), on the expression levels of different proteins (e.g. cyclin and CDKs), RNA levels (e.g. tyrosine kinase and phosphatases), and enzyme activity (e.g. DT-diaphorase and multidrug resistant - MDR). Microarray data measuring the baseline expression of thousands of genes is also available from two separate experiments. These molecular target data can be visualised in a Mean Graph and COMPARE can be used to identify possible correlations. In 1999, an *in vitro* pre-screen was introduced whereby compounds would be screened only in three highly sensitive cell lines: MCF-7 (breast ca), NCI-H460 (lung ca) and SF-268 (glioma). The rationale for introducing this pre-screen step was the finding that 85% of the compounds screened in the past had shown no evidence of anti-proliferative activity and this step would allow the removal of many inactive compounds from unnecessary and costly full scale evaluation in the 60-line panel.

The above described NCI screening programme is technically simple, relatively fast, cheap, reproducible and provides indicative data of mechanistic and target interaction. It is not, however, without false negative and false positive results. This screening was originally implemented to be sufficiently good to ensure that only a relatively small number of compounds would be selected for further evaluation in human tumour xenograft models. These cell screening assays are based on cells growing as monolayers. In the last decade many attempts have been made to develop 3D cell culture and co-culture systems to better mimic the structural and functional properties of normal and tumour tissue and to bridge the gap between cell-based assays and animal studies.[11,12] Their application to high throughput screening in drug development is, however, still to be validated.

2.1.2. *In vitro models to define the mechanisms of action of a given compound*

Once a compound has demonstrated robust cytotoxic activity against a panel of human cancer cell lines and deserves further investigation in *in vivo* models, it is important to clarify its mechanism of action and to identify its exact molecular targets. An example of the importance of the *in vitro* studies aimed at clarifying the mechanism of action of a given drug is represented by PARP inhibitors. Until a few years ago, PARP inhibitors had been thought of as agents to be used in combination with ionising radiation (IR) and with other different anticancer agents based on the assumption that they inhibited repair elicited by both IR and methylating agents. It was

later found that PARP inhibitors were very cytotoxic as single agents in tumour cells harbouring defects in recombination repair (i.e., cells with mutations of the genes *BRCA1* and *BRCA2*).[13,14] It was reported that both *BRCA1* and *BRCA2* homozygous mutant cells lines and tumour xenografts were hypersensitive to PARP inhibitor treatment, while wild type and heterozygous cell lines showed a normal sensitivity. As germ line mutations of *BRCA1* and *BRCA2* have been found to contribute to most of the familial breast cancer cases, the above mentioned exciting preclinical results opened new chemo-preventive and therapeutic strategies for *BRCA1/2*-associated breast cancers. The uses of isogenic cell lines with defined deficiency of each mechanism of DNA repair are available and very useful in defining the mode of action of new anticancer drugs.[15–17]

The recently put forward cancer stem cell hypothesis states that a minority of tumour cells (the cancer stem cells or tumour initiating cells - TIC - usually defined by cell surface marker expression) are responsible for tumour initiation, propagation and recurrence.[18–20] This hypothesis has important implications as the goal of the therapy is to specifically eradicate these cells.[21–23] The few data available seem to suggest that these cells are more radio and chemo-resistant than the remaining tumour cells. The detailed molecular and pharmacological characterisation of these TIC from different tumors (both leukaemias and solid tumours) will certainly be a useful tool not only to screen new compounds but also to identify new potential targets to be hit.

2.1.3. *The hollow fibre assay (HFA)*

The *in vivo* hollow fibre assay was developed at the NCI to help bridge the gap between the *in vitro* cell-based assays and human xenograft models in immuno-deficient mice.[24,25] The goal was to develop an intermediate assay that could better predict which compounds found active in the 60-cell line panel would be active in subsequent xenograft models. This was necessary due to the high cost of the traditional xenograft assay in terms of number of animals required, time for assay completion, and financial commitment necessary. To address this problem a short-term *in vivo* assay was devised in which tumour cells were grown in biocompatible hollow fibres for 24–48 h *in vitro*, followed by *in vivo* implantation at both intraperitoneal (i.p.) and subcutaneous (s.c.) sites of nude mice. These fibres have pores small enough to retain the cancer cells but large enough to permit entry of potential chemotherapeutic drugs (MW<500Kd), including peptides or large proteins. The predictive role of this assay has been recently reviewed and data suggest that the greater the response in the HFA, the greater the likelihood that a compound will be active in xenograft models. This prediction seems to be better if i.p. fibres are used. In addition, a strong correlation was reported between the activity in the 60-cell line screen and the HFA suggesting that this test is unlikely to miss active compounds at this preliminary *in vivo* stage of drug development.

The HFA has been more recently used to investigate the pharmacodynamics of anticancer agents *in vivo*.[26,27] Pharmacodynamic end points include protein/gene/mRNA expression, assessment of DNA damage (comet assay), cell cycle perturbation and cell death, including apoptosis. This approach allows a demonstration of an *in vivo* drug-target interaction and at the same time could eliminate those agents not shown to interact with their putative targets *in vivo* at an early preclinical stage.

2.2. *In vivo models*

The available *in vivo* models used to select compounds for further clinical development will be herein briefly summarised.

2.2.1. *Murine tumours*

P388 and L1210 leukaemia cell lines have been used for many years as the major preclinical models to screen new compounds.[28] They have been very useful in predicting the anti-leukaemic activity of many drugs that are still used for the therapy of rapidly growing tumours such as several haematological malignancies, but were not suitable models to identify compounds effective against solid tumours. Therefore, in the early 1970s, the B16 melanoma and Lewis lung carcinoma were also incorporated to try to identify compounds potentially active in human solid tumours. These tumours also induced the formation of lung metastases and thus were of potential use for investigating both the antitumour and the anti-metastatic activity of new compounds.

2.2.2. *Subcutaneously implanted human tumour xenografts*

Xenograft tumours have originally been established by inoculating s.c. tumour cells growing *in vitro* into nude mice which are immuno-deficient and allow the growth of a human tumour. Establishment of s.c. human tumours has been obtained both by inoculum of cells derived from human tumour cells cultured *in vitro* (the NCI made great efforts to obtain s.c. growing tumours from each of the 60 human cell lines used in the *in vitro* screening) and by direct implantation of patients tumour biopsy derived fragments or cells.[29–32] In this regard a particularly important large panel of xenografts has been established by Fiebig et al.[33,34] at the University of Frieburg in Germany. More than 1600 tumors have been transplanted s.c. into nude mice and more than 300 xenografts established, representative of all the major tumour types. In general, xenografts derived from patient biopsies retain better morphological and molecular properties reminiscent of the original human tumour as opposed to the ones derived from cultured cells that show a more homogeneous, undifferentiated histology probably indicative of the higher selection pressure *in vitro* during extensive culturing. Some disadvantages associated with xenografts obtained directly from patient biopsies are the high transplantation failure rate (the overall 'take rate' has been reported to vary from 40 to 60% for non-small cell lung cancer, colon cancer, and melanoma, down to 12–20%, for breast cancer and less than 3% for prostate cancer), the labour required for multiple transfers or 'passages' from mouse to mouse and the difficulty in establishing cell lines from them with scarce availability of pure human cells for biochemical and molecular studies. Not all xenografts models are usable for compound screening because of their growth properties. In fact sometimes growth is too slow (over many months) or too inconsistent/erratic/non linear with time and/or they possess cystic necrotic areas, even at relatively small tumour volumes. The s.c. implant allows the monitoring of tumour growth using *in situ* calipers and the activity of a

compound can be tested in different settings: agents can be administered at the same time as tumour implantation ('chemoprevention' strategy), treatment can start when tumours are just palpable ('early stage' strategy) although in such cases one needs to be aware that the residual immune capacity of the host (mainly natural killer activity) may influence the tumour response, and, finally, the treatment can start when tumour masses are bigger ('late stage' strategy). Activity is generally defined as tumour growth delay, optimal% T/C (median treated tumour mass/median control tumour mass expressed in %) or net log cell kill. Toxicity can be evaluated by monitoring body weight loss and drug-related deaths. Several studies have supported the value of the s.c. xenograft model in predicting the clinical activity of cytotoxic agents.[29,35–39] The NCI reported the results of a retrospective study in which the activity of 39 agents in both xenograft models and a phase II clinical trial were compared.[38] While the *in vivo* xenograft activity of a particular histology did not closely correlate with the activity in the same human cancer histology, a correlation was found for compounds active in at least 1/3 of xenografts that showed activity on some clinical trial. A better prediction of clinical outcome was found in the xenograft panel set up by Fiebig et al.[33,35] where the xenografts predicted correctly for clinical response in 90% of the cases and for resistance in 97%. In some cases the activity observed in xenografts is confirmed in the clinic but not necessarily in the same tumour type. In other cases the strong specificity for a tumour type has been instrumental for the undertaking of clinical investigations in that particular tumour type. For example, the striking antitumour activity of Trabectedin observed in well characterised ovarian cancer xenografts[40] provided the rationale for phase II,[41,42] and subsequently phase III, studies in this disease with very promising results.[43]

These predictions largely refer to the classical cytotoxic agents that have been developed in the last 25 years. Now many agents undergoing anticancer drug development are small molecules rationally designed to inhibit fundamental processes involved in the initiation and/or progression of human tumours. It can be said that the s.c. xenograft model can still be of value in the era of targeted therapy, if used appropriately however. It is becoming more and more important to molecularly characterise the different xenograft models to ensure that the putative molecular drug target is expressed and to integrate both pharmacokinetic and pharmacodynamic studies. Pharmacokinetics can give information on systemic exposure (area under the concentration-time curve; AUC) and comparing the systemic exposure associated with antitumour activity against human cancers in mice with the systemic drug exposure at the maximum tolerated dose (MTD) in patients may identify at a relatively early stage of development those agents that will ultimately fail in the clinic because it is impossible to achieve effective drug concentrations in humans.

2.2.3. *Orthotopic and metastatic tumour models*

Major drawbacks of the s.c. implanted human xenografts are that they grow in a tissue environment different from that of the organ they derive from and that they rarely metastasise. Orthotopic transplantation models were thought to mimic the morphology and growth characteristics of clinical disease and to better represent the clinical situation. Different orthotopic models have been obtained transplanting human colon carcinoma into the intestinal wall of nude mice, renal cancer into the kidney, melanoma into the skin, mammary carcinoma into mammary fad pad, bladder carcinoma into the bladder wall, prostate carcinoma into prostate, pancreatic carcinoma into the pancreas, and lung cancer into the bronchi. It has been demonstrated that the metastatic process is more efficient in orthotopically implanted tumours and that closely mimics human metastases. Some of them have been well characterised and are able to metastasise to clinical relevant sites, i.e. prostate and breast to the bone.[44–47] One of the most obvious advantages of the orthotopic models is the possibility to study and target processes involved in local invasion, e.g. inhibition of proteases and interfering with the process of angiogenesis. It is interesting to note that several reports have highlighted major differences in the therapeutic response between tumour transplanted s.c. and orthotopically. Kuo et al.[48] using an *in vivo* model of small-cell lung cancer showed that cisplatin had a significant effect against lung tumours but was ineffective against the same tumour transplanted s.c. On the contrary, the Fidler group reported that intestinal human cancer xenografts growing s.c. in nude mice respond to doxorubicin whereas the same tumour transplanted intracecally responded far less.[49] The recent development of imaging techniques allows a continuous evaluation of the size of the primary tumour and metastases, thus making it feasible to evaluate the kinetics of the drug effects by investigating a relatively small number of mice.[50]

2.2.4. *Genetically engineered cancer models (GEM)*

Over the past 20 years GEM models have been instrumental not only in our understanding of the molecular pathways responsible for the initiation and progression of tumours but also because they have highlighted the importance of specific oncogenes and tumour suppressor genes in carcinogenesis.[51,52] As GEM models have been shown to partially recapitulate the genetic/molecular changes occurring in human tumours, the challenge now is to use these models to test novel anticancer therapies in an attempt to better select clinically effective compounds.[30,53]

The initial GEMs were murine models that over-expressed viral and cellular oncogenes. With the discovery that loss of tumour suppressor gene (TSG) has a causative role in the development of tumours, transgenic mouse models were developed introducing a mutant TSG in the mouse germ line both by targeted gene knockout or through the expression of a dominant-negative form of the TSG. With both the improvement of molecular biology techniques and a better understanding of the biology of human tumours, it has been possible to develop mouse models carrying germ line mutation in specific genes, conditional transgenic/knockout models with spatiotemporal expression or loss of function of a specific TSG leading to the generation of many different GEM models that possess well validated drug targets; also, developing autochthonously *in situ* may probably be more biologically representative of a particular tumour type than transplanted xenografts.[54] In addition, GEM derived tumours develop in a setting of an intact immune system with a tumour-stromal interaction reproducing the one occurring in human tumours. However, their use in the preclinical devel-

opment of anticancer agents is still limited. These models are generally very expensive and time consuming and their use is often restricted by intellectual properties rights and patents. The spontaneous and multifocal nature of tumour development, the variable penetrance resulting in lack of synchrony in tumour development, the complicated breeding schemes, the difficulty in obtaining a sufficient number of animals at the same stage of tumour development, and the variability in time of progression to a predetermined tumour size required for drug testing (which can be measured in weeks-to-months with significant differences in age of animals and tumour stage in the same mouse colony) are all biological drawbacks preventing their widespread use. Although the use of GEMs in drug development has not been validated yet, different studies have suggested their potential utility[52,55–57] (Table 1). For example, retinoic acid has shown activity in GEMs of acute promyelocitic leukaemia[58] and imatinib has been shown to limit the development of bcr-abl mutation in p190 bcr-abl GEM mice.[59] In addition, GEM have also been used in a chemopreventing setting testing compounds that were shown to prevent the development of tumours.[60] A point that deserves to be outlined is the fact that the GEN models also provide primary cells and cell lines established from that can be used as a unique genetically defined tool for drug development.

The use of GEM models in drug discovery and development is, however, still in its infancy and only time and the analysis of the ongoing cumulating data on their use will tell if they will be any better in predicting clinical activity of anticancer agents than the xenograft models that are currently used in most cases.

3. Preclinical development of targeted agents

In the post-genomic era the focus of anticancer therapy has been the development of target therapy, where the target represents a molecular characteristic of the tumour that has a key role in its development and progression. In fact, the substantial advances made in understanding the basic biological principles underlying the genesis and progression of tumours have resulted in the identification of a number of molecular anomalies that are important not only for the malignant transformation of a normal cell but also for the maintenance of the tumour. The subsequent step was the development of

Table 1 – Examples of the use of GEM to test the activity of anticancer agents.

Mouse model	Drug used	Comments	Reference
PML-RARA transgenic mice developing APL	ATRA	ATRA treatment induced regression	Ref.[58,114]
PL2F-RARA transgenic mice developing APL	ATRA	ATRA treatment did not induce regression	Ref.[58]
Transgenic mouse models of breast cancer	Farnesyltransferase inhibitors (FTIs)	The data on these systems showed that the therapeutic effects of FTIs were due to off-target effects and not related to the inhibition of ras	Ref.[115–117]
Transgenic mice expressing exon 19 deletion mutant or the L856 mutant under the control of doxycicline	Erlotinib	Erlotinib treatment induced tumour regression	Ref.[118]
Bi-transgenic mice with inducible expression of two common hEGFR mutants	Erlotinib, HKI-272 and cetuximab	Erlotinib, HKI-272 and cetuximab treatment caused a dramatic tumour regression	Ref.[82]
Apc(Delta716) heterozygous mutant mouse, a model for human familial adenomatous polyposis	RAD001	RAD001 (everolimus) treatment suppressed the mTORC1 activity in the polyps and inhibited proliferation of the adenoma cells as well as tumour angiogenesis	Ref.[119]
Pancreatic islet carcinoma	Imatinib and SU11248 in combination with metronomic chemotherapy and/or VEGFR inhibition	Destabilisation of pre-existing tumour vasculature and inhibition of ongoing angiogenesis	Ref.[120]
TRAMP mice with prostate tumour	Silibinin	Silibinin treatment inhibited tumour growth, progression, local invasion, and distant metastasis involving suppression of tumour angiogenesis and epithelial-mesenchymal transition	Ref.[121]
	Recombinant adeno-associated virus-6 encoding mouse endostatin plus angiostatin (E + A) given i.m.	Antitumour activity observed	Ref.[122]
RIP-Tag2 transgenic mice with pancreatic cancer	Geldanamycin and/or 3-Bromo-pyruvate	Inhibition of pancreatic tumour and metastasis by more than 90% and significant prolongation of life span	Ref.[123]
Neuroblastoma in transgenic mice (TH-MYCN)	TNP-470	TNP-470 caused near-complete ablation, with reduced proliferation, enhanced apoptosis, and vasculature disruption	Ref.[124]

Table 2 – FDA approved targeted agents.

Drugs	Target	Type of molecule	Clinical development
Cetuximab (Erbitux)	EGFR	MoAb	FDA and EMEA approved (colorectal, head and neck ca)
Panitumumab (Vectibix)	EGFR	MoAb	FDA and EMEA approved (colorectal ca)
Trastuzumab (Herceptin)	HER2	MoAb	FDA and EMEA approved (HER2 overexpressing breast ca)
Erlotinib (Tarceva)	EGFR	SM	FDA and EMEA approved (NSCL, pancreatic)
Gefitinib (Iressa)	EGFR	SM	FDA and EMEA approved (NSCL)
Lapatinib (Tykerb)	EGFR, HER2	SM	Preregistration for breast cancer in European Union and USA, April 2009
Imatinib (Gleevec)	Bcl-Abl, c-Kit	SM	FDA and EMEA approved (chronic myeloid leukaemia, gastrointestinal stromal tumours)
Dasatinib (Sprycel)	BCR-ABL, SRC family, c-kit,EphA2, PDGFRß	SM	FDA and EMEA approved (Chronic myeloid leukaemia in all phases with resistance or intolerance to imatinib)
Bevacizumab (Avastin)	VEGF-a	MoAb	FDA and EMEA approved (glioblastoma, metastatic HER2 negative breast cancer, NSCL, metastatic colon ca)
Temsirolimus (Torisel)	mTOR	SM	FDA and EMEA approved (renal cell ca)
Sorafenib (Nexavar)	VEGFR, PDGFR, c-kit, Flt3, Raf,	SM	FDA and EMEA approved (hepatocellular carcinoma)
Sunitinib (Suten)	VEGFR, PDGFR, c-kit, Flt3	SM	FDA and EMEA approved (renal cell ca, gastrointestinal ca)
Vorinostat	HDAC inhibitor	Hydroxamic acid	FDA approved (cutaneous T cell lymphoma-third line therapy)

pharmacologic interventions targeting these molecular abnormalities leading to the so called targeted agents. Targeted therapy comprises mainly two classes of agents: small molecule inhibitors (mostly of tyrosine kinases) and monoclonal antibodies. Examples of aberrant targets that have been successfully exploited therapeutically include the epidermal growth factor (EGF) family of membrane receptors (including EGFR1, HER2), bcr-abl, CD20, c-Kit, components of the signal transduction network (e.g. mitogen-activated protein kinases-MAPK, PIK3 kinase transduction pathways, the mammalian target of rapamycin –mTOR), cell cycle regulators and factors involved in angiogenesis[61] (see Table 2).

This new class of targeted anticancer agents has clearly posed a different perspective to guide their preclinical and clinical development.[35,62,63] We will here try to summarise which are the key features to keep in mind when studying the preclinical activity of targeted agents and give some examples of what has been done with the recent FDA and European Medicines Agency (EMEA) approved targeted anticancer agents.

Once it has been established by biochemical assays that the drug is effective and sufficiently potent and specific for the target it has been designed against, the subsequent step is to verify that its biological activity is related to the functional inhibition of the target in different cellular contexts. This has been made possible by the availability of a number of antibodies that can either detect the inhibition of the target itself (i.e. inhibition of its autophosphorylation) or a downstream target; again, the specificity of the action of the drug also has to be confirmed in a cellular system. It is critical that the tumour model characterised for the relevant mutations or expression of the target protein is driven by, or at least expresses, the target of interest. Engineering tumours to be driven by the genetic changes of interest should be established in either mouse or human cells, when no tumour cells expressing the given target are available. While there are advantages to maintaining a syngenic relationship between tumour and host compartments, it is also important that the tumour target be human in origin. While for cytotoxic agents tumour cell killing was certainly one of the primary endpoints for *in vitro* and *in vivo* antitumour activity, in the current era of targeted therapy, inhibition of tumour growth may no longer be the only and most relevant endpoint. If a cancer target is well validated, an equally if not more relevant question is whether the therapeutic target is modulated *in vivo*. In fact, in the absence of the information that the drug is modulating the intended target, its antitumour efficacy is impossible to interpret. If, for example, an antitumour effect is observed and yet the intended target is not modulated, its observed antitumour activity may be the result of an off-target effect. Assessment of target modulation can be done by multiple methods sacrificing cohorts of mice over time and analysing the molecular target or a biomarker of a given pathway both in tumours or a surrogate organ (peripheral blood cells) using conventional biochemical and microscopic techniques (western blot, immuno-histochemistry). For example, the effects of a kinase inhibitor can be monitored by studying the activation state of the kinase using phospho-specific antibodies or downstream signalling mediators. In a time course experiment with the use of a different cohort of mice it will be possible to have a dynamic picture of the time necessary to inhibit the target and the long lasting effect of a given treatment on the target itself. Interestingly, an extension of the hollow fibre assay has been utilised to assess modulation of the molecular target.[27,64] Non invasive imaging has also been used to assess the molecular effects of drugs (i.e. molecular imaging) and this, in particular, is the case of antiangiogenic drugs.[65–67] By being non-invasive, molecular imaging can provide serial measurement over time in animals providing a true pharmacodynamic readout. Clinically useful functional imaging modalities have been adapted for small animal imaging, mainly rats and mice. These include micro-positron emission tomography (micro-PET) and magnetic resonance imaging (MRI), bioluminescence

and fluorescence imaging. For example, reporter cells can be engineered whereby luciferase expression is stimulus dependent and luminescence is the readout of the activity of a given molecular pathway.[68,69]

3.1. *Imatinib (Gleevec)*

Imatinib represents the best example of a drug that specifically targets the pathogenetic lesion of a human tumour such as chronic myelogenous leukaemia (CML). Bcl-abl is the chimeric product of a 9:22 chromosomal translocation in which *c-abl* translocates to chromosome 22 in a region known as the breakpoint cluster region (*bcr*).[70] Bcr-abl was shown to have an increased kinase activity as compared to c-abl and has been clearly demonstrated to be oncogenic. This translocation is present in more than 90% of CMLs and has been demonstrated to have a causative role in the development of CML. In the late 1980s, Ciba Geigy (now Novartis) started a high throughput screen of chemical libraries searching for compounds with kinase inhibitory activity.[71] A lead compound of the 2-phenylamminopyrimidine class was identified. It lacked the sufficient potency and specificity, but it represented the starting point for the synthesis of a series of related compounds that were optimised to inhibit a variety of targets. Imatinib (STI571, Gleevec) was found to inhibit abl, PDGRFs and KIT and emerged as the lead compound for preclinical development based on its selectivity against CML cells *in vitro* and its drug-like attributes, including pharmacokinetics and formulation properties.[72,73] Imatinib was shown to selectively suppress the proliferation of bcr-abl- expressing cells *in vitro* with minimal effect on normal bone marrow cells.[74] The selectivity was further confirmed in different laboratories and in many cells derived from CML and Ph+(Philadelphia Chromosome)ALL, while Ph- cells were unaffected. The *in vitro* IC_{50} for inhibition paralleled the IC_{50} for inhibition of bcr-abl kinase activity seen in cellular assays. A strong inhibitory effect on cellular growth was also observed in cells with activated deregulated PDGFR and c-KIT signalling. The *in vivo* activity of imatinib was tested on bcr-abl transformed 32D cells injected into syngenic mice.[74] A dose dependent inhibition of tumour growth could be observed after i.p. daily injections of the drug. A similar experiment using a bcr-abl + human cell line transplanted in nude mice demonstrated not only a good antitumour activity of the drug, but also the need for continuous inhibition of the bcr-abl kinase activity to achieve maximal antitumour effect. Pharmacokinetic studies in mice revealed that the compound after oral administration achieved pharmacologically relevant plasma concentrations but had a half life of approximately 1.5 h. When the schedule was optimised based on these data, (three times daily for 11 days), assuring a continuous blockage of the kinase, tumour-free survival of the mice could be registered. Again, this effect was specific as mice injected with U937 myeloid cells, leukaemia not expressing the bcr-abl fusion gene, and treated with this schedule had no observed growth inhibition. Based on its efficacy in the above mentioned preclinical models and an acceptable toxicological profile, phase I studies started on June 1998 and rapidly progressed to phase II studies demonstrating good activity that led to the approval by the FDA and EMEA for the treatment of CML in advanced stage and after failure on interferon therapy. It has been mentioned that imatinib also inhibits PDGFR and c-KIT. More than 90% of gastrointestinal stromal tumours (GIST) express KIT and the majority of them carry activating mutations.[75] In about 90% of cases the activation of KIT is due to a somatic mutation involving exon 9 or 11 and these mutations appear to be the critical pathogenetic event in GIST.[76] In addition, activation mutations of PDGRF are found in GIST. This tumour was considered an interesting clinical setting for testing imatinib activity. Thus there was a strong rationale to investigate the activity of imatinib in GIST and the clinical results were indeed very impressive, particularly considering that this tumour was considered refractory to the available therapies. A closer inspection of the data showed that the tumour KIT mutational status correlates with response: patients with mutated KIT had a 72% response rate while patients with no mutation had only an 18% response rate. Interestingly, one-third of the patients with no KIT mutation responding to imatinib were found to have PDGFRA mutations.[77] An important lesson that can be taken from the preclinical profiling of imatinib is the importance of having a robust and clinically relevant panel of cellular systems that can predict both cellular activity and selectivity. The preclinical experiments also defined the concentration of the drug required to obtain antitumour activity. The use of recombinant factor independent, bcr-abl transformed cell lines followed by testing on CML and normal patient samples correctly revealed the potential of imatinib and favoured its rapid movement to the clinic. This example is unique as bcr-abl has a causative role in CML development. Indeed, the presence of bcr-abl in the majority of CML patients and the possibility to identify patients carrying the bcr-abl oncogene by the presence of the Ph chromosome made it possible to easily select patients who were likely to benefit from imatinib therapy in early clinical trials. Another unique advantage that made the clinical development of the drug easier was the ability to monitor response with surrogate end-points, especially with haematological and cytogenetic response. In addition, it was also possible to incorporate a pharmacodynamic endpoint of inhibition of bcr-abl kinase activity by assaying CRKL phosphorylation. Lastly, from the pre-clinical studies, there was also an indication as to the threshold target levels of the drug (1 μM), a prediction that was indeed confirmed in clinical trials.

3.2. *Epidermal growth factor receptor (EGFR) targeted therapy*

EGFRs are receptor tyrosine kinases that play a key role in the regulation of essential normal cellular processes and in the patho-physiology of hyperproliferative disease, including cancer.[77] Four members belong to this family: EGFR, HER2, HER3, and HER4. Preclinical studies conducted over the last 20 years have clearly demonstrated that alterations in the function of these membrane receptors, particularly EGFR, are associated with oncogenic transformation and that deregulation of EGFR is related to all the key features of cancer: autonomous cell growth, invasion, angiogenesis potential and development of distant metastasis. Activation of EGFR leads to different intracellular signalling pathways including the RAS/RAF/MAPK-mediated pathway leading to proliferation

and the PI3 K/AKT pathways which increase survival by anti-apoptotic mechanisms. As a consequence, therapeutics that target this family of receptors have been developed.

Two classes of molecules have been developed: monoclonal antibodies (MAbs) that bind to the extracellular part of the receptor and prevent binding of the natural ligands and inhibitors of tyrosine kinase that are small molecules (SMs) that compete with and prevent binding of the ATP to the intracellular tyrosine kinase region.[78] In both cases preclinical development has been aimed at demonstrating that the compounds bind to the receptor and interfere with its signalling transduction pathways. The binding affinity values and the IC_{50} values inhibiting the kinase activity of the tyrosine kinase were determined and whenever possible compared with the values obtained with drugs of the same class of agents. The specificity of action has to be considered when testing the activity of the compound against a panel of different kinases important for cancer either in purified enzyme assays and/or in whole cell extracts. The inhibition of the auto-phosphorylation of EGFR, coupled with its internalisation and degradation (in the case of MAbs), had to be demonstrated for all the compounds as well as the functional consequence of the inhibition of the signalling transduction pathways. With both MAbs and SMs, the inhibition of the receptor in different cellular systems expressing the receptor has been shown to be related to an inhibition of cell cycle progression by the induction of the cell cycle regulator p27 and induction of apoptosis by either increasing the expression of pro-apoptotic protein or by the inactivation of anti-apoptotic protein through decreased expression or phosphorylation. The *in vivo* antitumour effect of such compounds has been tested in xenograft models and their antitumour activity related to the inhibition of the target.[79] Cetuximab showed antitumour activity against a variety of human tumour xenografts and also displayed synergistic effects when combined with classical anticancer agents. In the case of panitumumab, *in vivo* treatment efficacy in xenografts appeared to be associated with EGFR expression levels. Preclinical studies carried out with erlotinib showed a direct relationship between target inhibition and its antitumour effects, suggesting that sufficient doses to inhibit the target in tumour tissues would need to be administered for this agent to be effective in the clinic. When gefitinib was tested in human xenografts it showed a marked antitumour activity with rapid regression of established tumours but no clear cut correlation with the expression of EGFR, suggesting that other factors besides EGFR expression may be involved in tumour response to the drug.[63] When tested in a clinical setting these EGFR target agents did show some clinical activity and some have been approved by the FDA and EMEA for some specific tumour settings (see Table 2). The original prediction that only patients over-expressing EGFR would benefit from treatment with these agents was not fulfilled as several clinical studies suggest that EGFR expression is not sufficient to predict response to treatment.[80,81] It has in fact been reported that NSCLC patients, non-smokers harbouring some mutations in the ATP pocket, were more responsive to gefitinib or erlotinib. It was later demonstrated that this mutation had an oncogenic role in lung tumourigenesis.[82] Recent data suggest that a T790 M mutation (resulting in a threonine to methionine substitution at amino acid position 790) could emerge during treatment with gefitinib and experimental data corroborated the fact that cells over-expressing this mutation were highly resistant to the drug.[83] Several other mechanisms of pathway activation have been put forward in recent years such as gene amplification, amplification of a dinucleotide repeat in the EGFR promoter, mutation in the extracellular region of EGFR giving rise to a mutant protein named EGFRvIII and enhanced signalling due to heterodimerisation with other EGFR members (HER2 and HER3), and interestingly enough an association has been found with the clinical activity of the drug. Retrospective analysis of tumour samples also demonstrated that, in colon cancer patients, mutation in K-RAS was associated with a lack of response and reduced survival after treatment with cetuximab.[84–86]

3.3. *Trastuzumab*

Trastuzumab is a humanised monoclonal antibody directed against the HER2 receptor.[87] It has been initially approved by the FDA for the treatment of HER2 positive metastasised breast cancer after treatment failure with other anticancer agents; in 2006 the FDA granted approval to trastuzumab as part of a treatment regimen containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant setting in women with node-positive, HER2 over-expressing tumours based on the data of increased disease free survival in women who received chemotherapy plus trastuzumab compared to those who received chemotherapy alone.[88,89] HER was identified as a valid target as it was demonstrated, using transgenic mice, to be oncogenic and to have a role in the pathogenesis of breast cancer.[90] The *in vitro* data demonstrated that binding of trastuzumab to HER2 resulted in attenuation of aberrant HER2 kinase-associate signal transduction, with changes in cell cycle distribution (e.g. decreases in the fraction of cells undergoing S-phase) specifically in cells over-expressing the target.[86] When the antibody was tested in an *in vivo* setting, it seemed reasonable to select breast cancer xenografts that were expressing HER2. However, all the commercially available cell lines over- or expressing the target did not grow subcutaneously in nude mice.[91] Artificially engineered models had to be set up transfecting human cell lines known to be tumourigenic in nude mice with HER cDNA. These over-expressing HER2 models, although clearly artificial and not directly related to the human disease, were invaluable in the preclinical development of trastuzumab as it facilitated the *in vivo* testing of the antibody. Another approach undertaken was the use of fresh patients' tumour explants from HER2-positive tumours implanted directly into the subrenal capsule of athymic mice.[92] In this assay, size determinations of tumour transplants were performed and the observed decreases in tumour sizes relative to untreated controls were considered evidence for *in vivo* anti-tumour activity and this helped in deciding which murine anti-HER2 antibody to humanise for future clinical development. This kind of assay did, however, have limitations such as the availability of fresh tumour implants, the tumour take-up rates (generally between 60 and 80%) and the relative small number of animals in each treatment group. Also instrumental were the studies performed with radio-labelled anti-HER2 antibody, that

unambiguously demonstrated the uptake and the retention of the radio-labelled Ab in tumour deposits *in vivo*. The above mentioned overexpressing-HER2 cell lines transplanted in nude mice were utilised to demonstrate a clear *in vivo* dose–response relationship for trastuzumab and to correlate response with mouse serum pharmacokinetics. These studies allowed the definition of the minimum target serum concentration for trastuzumab necessary for its antitumour efficacy before the initiation of the first phase I clinical trial. The principal shortcoming of animal modelling in the development of trastuzumab was probably the lack of cross reactivity of trastuzumab to non-human HER2. Thus, it was difficult, if not impossible, to predict unanticipated toxicities of trastuzumab, such as cardiac dysfunction. A potentially important mechanism of immunologic activity of therapeutic antibodies is the antibody-dependent cell-mediated cytotoxicity (ADCC) resulting from activation of Fc receptors on immune effector cells and polymorphisms in these genes have been associated with a different efficiency in the ADCC. A role for ADCC in the mechanism of action of trastuzumab has been suggested by several lines of evidence.[93,94] ADCC activity was in fact demonstrated *ex vivo* in peripheral blood mononuclear cells isolated from patients with metastatic breast cancer undergoing treatment with trastuzumab. An increase in trastuzumab anti-tumour activity against breast cancer xenografts in knockout mouse models in which inhibitory Fc receptors (FcRII) are lacking has been shown and attenuated trastuzumab responses in knockout mice lacking activating FcRIII receptors have been reported. Finally, administration of an anti-HER2 antibody mutant construct lacking functional FcR binding capability similarly has marked attenuation of antitumour activity in xenograft models. All these data support not only a clear role in ADCC in the antitumour effect of trastuzumab but also how the use of mice has been instrumental in clarifying such a mechanism.

3.4. *Agents targeting angiogenesis*

The dependency of tumour growth on the development of a neovasculature is a well established aspect of cancer biology and it is now recognised that inhibition of angiogenesis can be a strategy for inhibiting tumour growth and metastasis.[95,96] Two classes of agents interfering with this process have been identified: anti-angiogenic agents that prevent the formation of tumour blood vessels and vascular-targeting agents that target the existing tumour vessels.[97] Included in the former class are bevacizumab (a humanised monoclonal antibody directed against vascular endothelial growth factor [VEGF]), sunitib and sorafenib (small molecules inhibiting tyrosine kinase receptor VEGFR) which have obtained FDA approval for a number of different indications (Table 2). The preclinical development of this class of compounds has been based on a number of assays that mimic the different steps of the angiogenic process.[98] The knowledge of the putative mechanism of action of the selected compound can dictate the choice of assay; on the contrary, the behaviour of the compound in a panel of different assays could suggest its mechanism of action. The *in vitro* tests usually rely on the use of normal endothelial cells (the most commonly used are the ones from the human umbilical vein origin); less common are the tumour-derived endothelial cells which would probably be more representative but technical problems limit their use. The inhibition of their proliferation, the induction of apoptosis, the inhibition of their motility, of their capacity to degrade the extracellular matrix and the ability to form cord or tube *in vitro* can be quantified in well validated assays. For the technical and detailed description of all these tests please refer to the references herein.[99–102] Several *in vivo* assays have been set up to allow the rapid, reproducible and, possibly, quantitative effect of antiangiogenic componds: the directed *in vivo* angiogenic assay, the chick chorioallantoic membrane (CAM) method, the matrig plug assay, the cornea assay and the chronic transparent chamber and intravital microscopy. Depending on the test, the detection and evaluation can be done throughout the duration of the experiment (cornea and artificial chamber) or else the evaluation can be done at just one time point. Nevertheless, even for this class of compounds, the final validation in *in vivo* tumour model was required. The most commonly used tumour models were the Lewis lung carcinoma, that has the advantage of growing in a syngenic mouse with an intact host response, and different human xenografts transplanted both subcutaneously and orthotopically. More recently, angiogenesis dependent tumours, in which the expression of different angiogenic factors can be modulated, and GEM models, which allow the study of the antiangiogenic effects at all stages of tumour progression, have also been used to explore the anti-tumour effect of the angiogenic agents.[103–106] In addition, a number of pharmacodynamic end points have been studied to prove the effect of these agents on tumour vasculature such as the immuno-staining of endothelial cells by different antibodies (CD31), measurement of tumour vessel structure, dimension and dilatation, vessel maturation, permeability, measurement of different metabolic parameters and oxygen tension in tumour specimens.[107,108] More recently, the development of new imaging techniques (the application of MRI and PET to small animals such as mice and rats) has allowed the study of the response of tumours at different time points after the administration of anti-angiogenic therapy.[109]

4. Concluding remarks

The preclinical experimental models that are currently used for the identification and selection of novel anticancer drugs, which were overviewed in the present paper, are far from being satisfactory in mimicking the complex biological features of human tumours. Nevertheless, these experimental models have been instrumental in the successful clinical development of many anticancer drugs, including many natural products and synthetic compounds directed to DNA, chromatin, and tubulin as well as antibodies and small molecules directed to different kinases involved in pathways that are relevant for cancer cell survival, growth, differentiation and tumour angiogenesis. However, the enormous advancement of knowledge in tumour biology together with the identification of hundreds of molecular targets suitable for pharmacological intervention has, so far, not resulted in the expected improvement of the efficacy of cancer therapy. It should be pointed out that cancer comprises hundreds of

different diseases; each of them is remarkably heterogeneous in different patients and even in the same patient during the progression of the disease.

In the recent characterisation of the genome of human tumours[110,111], it has become clear that each tumour has several dozen point mutations, gene deletions and amplifications. Therefore, to reproduce in preclinical models the complexity of human cancer is unfeasible. It is of note, however, that the genetic alterations are related to a limited number of pathways that are aberrantly activated.[15,112] Therefore, the development of preclinical models that are representative of the deregulation of specific pathways may be relevant for testing novel drugs, this being a much more feasible task.

We would like to stress that there are no ideal models that can be used for any kind of drug, but depending on the drug the most suitable experimental models should be selected. For example, for compounds with unknown modes of action, such as natural products identified in cellular screenings, it seems sensible to use a variety of highly characterised human xenografts that will possibly provide indications on their therapeutic index, mode of action and the determinants of their antitumour effects. Well characterised xenografts obtained by transplantation of human tumour fragments maintain, in many cases, some of the original pathological and biological features for a few passages,[113] thus being potentially useful to investigate specific classes of compounds interfering with pathways that are aberrantly activated in human tumours. Molecularly characterised xenografts are also potentially useful for pharmacokinetic/pharmacodynamic studies and to discover potential markers to monitor pharmacological effects. Some xenografts induce the formation of metastases when orthotopically transplanted, thus making it possible to evaluate potential anti-metastatic therapies. These models, adequately characterised, can also be useful in evaluating anti-angiogenic and anti-vascular therapies, previously tested on *in vitro* and *in vivo* angiogenesis assays. Genetic models are particularly relevant for the validation of the potential therapeutic value of new targets. The possibility to up- or down-regulate a given enzyme or a pathway provides an anticipation of the effects of compounds acting by these mechanisms. These models, which although in principle are the ideal ones for investigating novel target therapies, are in most cases of limited value for a quantitative statistical evaluation of the antitumour effects of a new drug because of their very variable growth in mice.

In order to investigate potential drugs which hit specific molecular targets, it is very important that biologists, who have a deep knowledge of the target, do participate in the selection of the most suitable system to test compounds directed against that target. Deep knowledge of the target is important for making suggestions to the chemists, indicating what should be the optimal features of potential inhibitors. In addition, it is important that medical oncologists take part in the preclinical development of drugs to critically evaluate the clinical relevance of the preclinical experimental models, to investigate potential biomarkers, to monitor drug therapies, and to plan the early clinical investigations in the safest and most rational ways. The role of pharmacologists and toxicologists is essential to optimise treatments according to the pharmacokinetic/pharmacodynamic characteristics of the new compounds and to translate this knowledge to appropriate clinical protocols.

In summary, the development of new drugs requires an inter-disciplinary approach that allows consideration of all the aspects of the biology, chemistry and pharmacology of a new agent, as well as the specific oncological knowledge, in order to direct the preclinical–clinical development in the most rational way. Certainly, the huge number of new targets and new molecules requires a continuous updating of the experimental models suitable for optimal selection of any new compound with a novel mode of action for clinical investigation. Therefore, the development of preclinical experimental models still represents a challenge for any scientist working in this field.

Conflict of interest statement

None declared.

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REFERENCES

1. Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. *CA Cancer J Clin* 2009;**59**:111–37.
2. Morgan TM, Koreckij TD, Corey E. Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. *Curr Cancer Drug Targets* 2009;**9**:237–49.
3. Hait WN, Hambley TW. Targeted cancer therapeutics. *Cancer Res* 2009;**69**:1263–7 [discussion 1267].
4. Rutkowski P, Ruka W. Emergency surgery in the era of molecular treatment of solid tumours. *Lancet Oncol* 2009;**10**:157–63.
5. Lorusso G, Ruegg C. The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochem Cell Biol* 2008;**130**:1091–103.
6. Suggitt M, Bibby MC. 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. *Clin Cancer Res* 2005;**11**:971–81.
7. Holbeck SL. Update on NCI in vitro drug screen utilities. *Eur J Cancer* 2004;**40**:785–93.
8. Paull KD, Shoemaker RH, Hodes L, et al. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. *J Natl Cancer Inst* 1989;**81**:1088–92.
9. Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. Proteasome inhibition as a novel therapeutic target in human cancer. *J Clin Oncol* 2005;**23**:630–9.
10. Sausville EA, Feigal E. Evolving approaches to cancer drug discovery and development at the National Cancer Institute, USA. *Ann Oncol* 1999;**10**:1287–91.
11. Friedrich J, Ebner R, Kunz-Schughart LA. Experimental anti-tumor therapy in 3-D: spheroids–old hat or new challenge? *Int J Radiat Biol* 2007;**83**:849–71.

12. Lin RZ, Chang HY. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 2008;**3**:1172–84.
13. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005;**434**:913–7.
14. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005;**434**:917–21.
15. Sharma SV, Settleman J. Oncogene addiction: setting the stage for molecularly targeted cancer therapy. *Genes Dev* 2007;**21**:3214–31.
16. Damia G, D'Incalci M. Targeting DNA repair as a promising approach in cancer therapy. *Eur J Cancer* 2007;**43**:1791–801.
17. Tavecchio M, Simone M, Erba E, et al. Role of homologous recombination in trabectedin-induced DNA damage. *Eur J Cancer* 2008;**44**:609–18.
18. Besancon R, Valsesia-Wittmann S, Puisieux A, de Fromentel CC, Maguer-Satta V. Cancer stem cells: the emerging challenge of drug targeting. *Curr Med Chem* 2009;**16**:394–416.
19. Marotta LL, Polyak K. Cancer stem cells: a model in the making. *Curr Opin Genet Dev* 2009;**19**:44–50.
20. Finlan LE, Hupp TR. Epidermal stem cells and cancer stem cells: insights into cancer and potential therapeutic strategies. *Eur J Cancer* 2006;**42**:1283–92.
21. Park CY, Tseng D, Weissman IL. Cancer stem cell-directed therapies: recent data from the laboratory and clinic. *Mol Ther* 2009;**17**:219–30.
22. Blagosklonny MV. Target for cancer therapy: proliferating cells or stem cells. *Leukemia* 2006;**20**:385–91.
23. Simeone DM. Pancreatic cancer stem cells: implications for the treatment of pancreatic cancer. *Clin Cancer Res* 2008;**14**:5646–8.
24. Decker S, Hollingshead M, Bonomi CA, Carter JP, Sausville EA. The hollow fibre model in cancer drug screening: the NCI experience. *Eur J Cancer* 2004;**40**:821–6.
25. Hall LA, Krauthauser CM, Wexler RS, Hollingshead MG, Slee AM, Kerr JS. The hollow fiber assay: continued characterization with novel approaches. *Anticancer Res* 2000;**20**:903–11.
26. Suggitt M, Cooper PA, Shnyder SD, Bibby MC. The hollow fibre model–facilitating anti-cancer pre-clinical pharmacodynamics and improving animal welfare. *Int J Oncol* 2006;**29**:1493–9.
27. Temmink OH, Prins HJ, van Gelderop E, Peters GJ. The Hollow Fibre Assay as a model for in vivo pharmacodynamics of fluoropyrimidines in colon cancer cells. *Br J Cancer* 2007;**96**:61–6.
28. Venditti JM, Wesley RA, Plowman J. Current NCI preclinical antitumor screening in vivo: results of tumor panel screening, 1976–1982, and future directions. *Adv Pharmacol Chemother* 1984;**20**:1–20.
29. Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer* 2004;**40**:827–36.
30. Talmadge JE, Singh RK, Fidler IJ, Raz A. Murine models to evaluate novel and conventional therapeutic strategies for cancer. *Am J Pathol* 2007;**170**:793–804.
31. Fiebig HH, Maier A, Burger AM. Clonogenic assay with established human tumour xenografts: correlation of in vitro to in vivo activity as a basis for anticancer drug discovery. *Eur J Cancer* 2004;**40**:802–20.
32. Alley MC, Scudiero DA, Monks A, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 1988;**48**:589–601.
33. Garber K. From human to mouse and back: 'tumorgraft' models surge in popularity. *J Natl Cancer Inst* 2009;**101**:6–8.
34. Boven E, Winograd B, Berger DP, et al. Phase II preclinical drug screening in human tumor xenografts: a first European multicenter collaborative study. *Cancer Res* 1992;**52**:5940–7.
35. Fiebig HH, Berger DP, Winterhalter BR, Plowman J. In vitro and in vivo evaluation of US-NCI compounds in human tumor xenografts. *Cancer Treat Rev* 1990;**17**:109–17.
36. Sausville EA, Burger AM. Contributions of human tumor xenografts to anticancer drug development. *Cancer Res* 2006;**66**:3351–4 [discussion 3354].
37. Steel GG, Courtenay VD, Peckham MJ. The response to chemotherapy of a variety of human tumour xenografts. *Br J Cancer* 1983;**47**:1–13.
38. Johnson JI, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br J Cancer* 2001;**84**:1424–31.
39. Kerbel RS. Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved. *Cancer Biol Ther* 2003;**2**:S134–9.
40. Valoti G, Nicoletti MI, Pellegrino A, et al. Ecteinascidin-743, a new marine natural product with potent antitumor activity on human ovarian carcinoma xenografts. *Clin Cancer Res* 1998;**4**:1977–83.
41. Sessa C, De Braud F, Perotti A, et al. Trabectedin for women with ovarian carcinoma after treatment with platinum and taxanes fails. *J Clin Oncol* 2005;**23**:1867–74.
42. Krasner CN, McMeekin DS, Chan S, et al. A Phase II study of trabectedin single agent in patients with recurrent ovarian cancer previously treated with platinum-based regimens. *Br J Cancer* 2007;**97**:1618–24.
43. Krasner CN, Poveda A, Herzog T, et al. Health-related quality of life/patient-reported outcomes in relapsed ovarian cancer: Results from a randomized phase III study of trabectedin with pegylated liposomal doxorubicin (PLD) versus PLD alone. *J Clin Oncol* 2009:27 [abstract 5526].
44. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* 2004;**40**:852–7.
45. Hoffman RM. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. *Invest New Drugs* 1999;**17**:343–59.
46. Killion JJ, Radinsky R, Fidler IJ. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev* 1998;**17**:279–84.
47. Hoffman RM. Orthotopic metastatic (MetaMouse) models for discovery and development of novel chemotherapy. *Methods Mol Med* 2005;**111**:297–322.
48. Kuo TH, Kubota T, Watanabe M, et al. Site-specific chemosensitivity of human small-cell lung carcinoma growing orthotopically compared to subcutaneously in SCID mice. the importance of orthotopic models to obtain relevant drug evaluation data. *Anticancer Res* 1993;**13**:627–30.
49. Fidler IJ, Wilmanns C, Staroselsky A, Radinsky R, Dong Z, Fan D. Modulation of tumor cell response to chemotherapy by the organ environment. *Cancer Metastasis Rev* 1994;**13**:209–22.
50. Olive KP, Tuveson DA. The use of targeted mouse models for preclinical testing of novel cancer therapeutics. *Clin Cancer Res* 2006;**12**:5277–87.
51. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer* 2007;**7**:645–58.
52. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* 2006;**5**:741–54.
53. Robles AI, Varticovski L. Harnessing genetically engineered mouse models for preclinical testing. *Chem Biol Interact* 2008;**171**:159–64.
54. Van Dyke T, Jacks T. Cancer modeling in the modern era: progress and challenges. *Cell* 2002;**108**:135–44.

55. Singh M, Johnson L. Using genetically engineered mouse models of cancer to aid drug development: an industry perspective. *Clin Cancer Res* 2006;**12**:5312–28.
56. Gutmann DH, Hunter-Schaedle K, Shannon KM. Harnessing preclinical mouse models to inform human clinical cancer trials. *J Clin Invest* 2006;**116**:847–52.
57. Varticovski L, Hollingshead MG, Robles AI, et al. Accelerated preclinical testing using transplanted tumors from genetically engineered mouse breast cancer models. *Clin Cancer Res* 2007;**13**:2168–77.
58. Rego EM, He LZ, Warrell Jr RP, Wang ZG, Pandolfi PP. Retinoic acid (RA) and As_2O_3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARalpha and PLZF-RARalpha oncoproteins. *Proc Natl Acad Sci USA* 2000;**97**:10173–8.
59. Brain J, Saksena A, Laneuville P. The kinase inhibitor STI571 reverses the Bcr-Abl induced point mutation frequencies observed in pre-leukemic P190(Bcr-Abl) transgenic mice. *Leuk Res* 2002;**26**:1011–6.
60. Boolbol SK, Dannenberg AJ, Chadburn A, et al. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res* 1996;**56**:2556–60.
61. Press MF, Lenz HJ. EGFR, HER2 and VEGF pathways: validated targets for cancer treatment. *Drugs* 2007;**67**:2045–75.
62. Kung AL. Practices and pitfalls of mouse cancer models in drug discovery. *Adv Cancer Res* 2007;**96**:191–212.
63. Kornek G, Selzer E. Targeted therapies in solid tumours: pinpointing the tumour's Achilles heel. *Curr Pharm Des* 2009;**15**:207–42.
64. Shnyder SD, Hasan J, Cooper PA, et al. Development of a modified hollow fibre assay for studying agents targeting the tumour neovasculature. *Anticancer Res* 2005;**25**:1889–94.
65. Riemann B, Schafers KP, Schober O, Schafers M. Small animal PET in preclinical studies: opportunities and challenges. *Q J Nucl Med Mol Imaging* 2008;**52**:215–21.
66. Rowland DJ, Cherry SR. Small-animal preclinical nuclear medicine instrumentation and methodology. *Semin Nucl Med* 2008;**38**:209–22.
67. Sundaram B, Quint LE, Patel HJ, Deeb GM. CT findings following thoracic aortic surgery. *Radiographics* 2007;**27**:1583–94.
68. Villalobos V, Naik S, Piwnica-Worms D. Current state of imaging protein-protein interactions in vivo with genetically encoded reporters. *Annu Rev Biomed Eng* 2007;**9**:321–49.
69. Hwang do W, Kang JH, Jeong JM, et al. Noninvasive in vivo monitoring of neuronal differentiation using reporter driven by a neuronal promoter. *Eur J Nucl Med Mol Imaging* 2008;**35**:135–45.
70. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 1973;**243**:290–3.
71. Lydon NB, Druker BJ. Lessons learned from the development of imatinib. *Leuk Res* 2004;**28**(Suppl 1):S29–38.
72. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005;**105**:2640–53.
73. Mauro MJ, O'Dwyer M, Heinrich MC, Druker BJ. STI571: a paradigm of new agents for cancer therapeutics. *J Clin Oncol* 2002;**20**:325–34.
74. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 1996;**2**:561–6.
75. Tornillo L, Terracciano LM. An update on molecular genetics of gastrointestinal stromal tumours. *J Clin Pathol* 2006;**59**:557–63.
76. Sleijfer S, Wiemer E, Verweij J. Drug Insight: gastrointestinal stromal tumors (GIST)–the solid tumor model for cancer-specific treatment. *Nat Clin Pract Oncol* 2008;**5**:102–11.
77. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* 2009;**21**:177–84.
78. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005;**5**:341–54.
79. Grunwald V, Hidalgo M. Developing inhibitors of the epidermal growth factor receptor for cancer treatment. *J Natl Cancer Inst* 2003;**95**:851–67.
80. Laurent-Puig P, Lievre A, Blons H. Mutations and response to epidermal growth factor receptor inhibitors. *Clin Cancer Res* 2009;**15**:1133–9.
81. Hopper-Borge EA, Nasto RE, Ratushny V, Weiner LM, Golemis EA, Astsaturov I. Mechanisms of tumor resistance to EGFR-targeted therapies. *Expert Opin Ther Targets* 2009;**13**:339–62.
82. Ji H, Li D, Chen L, et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* 2006;**9**:485–95.
83. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;**359**:366–77.
84. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009;**27**:2091–6.
85. Lievre A, Bachet JB, Boige V, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008;**26**:374–9.
86. De Roock W, Piessevaux H, De Schutter J, et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 2008;**19**:508–15.
87. Cho HS, Mason K, Ramyar KX, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 2003;**421**:756–60.
88. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 2005;**353**:1659–72.
89. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005;**353**:1673–84.
90. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–37.
91. Pegram M, Ngo D. Application and potential limitations of animal models utilized in the development of trastuzumab (Herceptin): a case study. *Adv Drug Deliv Rev* 2006;**58**:723–34.
92. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992;**89**:4285–9.
93. Arnould L, Gelly M, Penault-Llorca F, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br J Cancer* 2006;**94**:259–67.
94. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* 2008;**26**:1789–96.
95. Cao Y. Tumor angiogenesis and molecular targets for therapy. *Front Biosci* 2009;**14**:3962–73.

96. Cao Y, Langer R. A review of Judah Folkman's remarkable achievements in biomedicine. *Proc Natl Acad Sci USA* 2008;**105**:13203–5.
97. Ribatti D. The discovery of antiangiogenic molecules: a historical review. *Curr Pharm Des* 2009;**15**:345–52.
98. Cao Y. Molecular mechanisms and therapeutic development of angiogenesis inhibitors. *Adv Cancer Res* 2008;**100**:113–31.
99. D'Onofrio A, Gandolfi A. A family of models of angiogenesis and anti-angiogenesis anti-cancer therapy. *Math Med Biol* 2009;**26**:63–95.
100. Taraboletti G, Giavazzi R. Modelling approaches for angiogenesis. *Eur J Cancer* 2004;**40**:881–9.
101. MacDonald IC, Chambers AF. Chapter 9. Intravital videomicroscopy in angiogenesis research. *Methods Enzymol* 2008;**444**:201–30.
102. Vargas A, Zeisser-Labouebe M, Lange N, Gurny R, Delie F. The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems. *Adv Drug Deliv Rev* 2007;**59**:1162–76.
103. Dalal S, Burchill SA. Preclinical evaluation of vascular-disrupting agents in Ewing's sarcoma family of tumours. *Eur J Cancer* 2009;**45**:713–22.
104. Lee FY, Covello KL, Castaneda S, et al. Synergistic antitumor activity of ixabepilone (BMS-247550) plus bevacizumab in multiple in vivo tumor models. *Clin Cancer Res* 2008;**14**:8123–31.
105. Ogata T, Kurabayashi M, Maeno T, et al. An angiogenesis inhibitor TNP-470 (AGM-1470) suppresses vascular smooth muscle cell proliferation in experimental rat aortotomy models. *J Cardiovasc Surg (Torino)* 2004;**45**:497–500.
106. Zhang C, Yan Z, Arango ME, Painter CL, Anderes K. Advancing bioluminescence imaging technology for the evaluation of anticancer agents in the MDA-MB-435-HAL-Luc mammary fat pad and subrenal capsule tumor models. *Clin Cancer Res* 2009;**15**:238–46.
107. Brown JQ, Wilke LG, Geradts J, Kennedy SA, Palmer GM, Ramanujam N. Quantitative optical spectroscopy: a robust tool for direct measurement of breast cancer vascular oxygenation and total hemoglobin content in vivo. *Cancer Res* 2009;**69**:2919–26.
108. Fox SB. Assessing tumor angiogenesis in histological samples. *Methods Mol Biol* 2009;**467**:55–78.
109. Feng Y, Jeong EK, Mohs AM, Emerson L, Lu ZR. Characterization of tumor angiogenesis with dynamic contrast-enhanced MRI and biodegradable macromolecular contrast agents in mice. *Magn Reson Med* 2008;**60**:1347–52.
110. Mullighan CG, Downing JR. Global genomic characterization of acute lymphoblastic leukemia. *Semin Hematol* 2009;**46**:3–15.
111. Fox EJ, Salk JJ, Loeb LA. Cancer genome sequencing–an interim analysis. *Cancer Res* 2009;**69**:4948–50.
112. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**:57–70.
113. Fichtner I, Slisow W, Gill J, et al. Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas. *Eur J Cancer* 2004;**40**:298–307.
114. He LZ, Guidez F, Tribioli C, et al. Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat Genet* 1998;**18**:126–35.
115. Lobell RB, Omer CA, Abrams MT, et al. Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. *Cancer Res* 2001;**61**:8758–68.
116. Kohl NE, Omer CA, Conner MW, et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nat Med* 1995;**1**:792–7.
117. Omer CA, Chen Z, Diehl RE, et al. Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor. *Cancer Res* 2000;**60**:2680–8.
118. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev* 2006;**20**:1496–510.
119. Fujishita T, Aoki K, Lane HA, Aoki M, Taketo MM. Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in ApcDelta716 mice. *Proc Natl Acad Sci USA* 2008;**105**:13544–9.
120. Pietras K, Hanahan D. A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. *J Clin Oncol* 2005;**23**:939–52.
121. Singh RP, Raina K, Sharma G, Agarwal R. Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice. *Clin Cancer Res* 2008;**14**:7773–80.
122. Isayeva T, Chanda D, Kallman L, Eltoum IE, Ponnazhagan S. Effects of sustained antiangiogenic therapy in multistage prostate cancer in TRAMP model. *Cancer Res* 2007;**67**:5789–97.
123. Cao X, Jia G, Zhang T, et al. Non-invasive MRI tumor imaging and synergistic anticancer effect of HSP90 inhibitor and glycolysis inhibitor in RIP1-Tag2 transgenic pancreatic tumor model. *Cancer Chemother Pharmacol* 2008;**62**:985–94.
124. Chesler L, Goldenberg DD, Seales IT, et al. Malignant progression and blockade of angiogenesis in a murine transgenic model of neuroblastoma. *Cancer Res* 2007;**67**:9435–42.