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Preclinical predictors of anticancer drug efficacy: Critical assessment with emphasis on whether nanomolar potency should be required of candidate agents

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Abbreviations: ADME, absorption, distribution, metabolism and excretion; COX, cyclooxygenase; FDA, US Food and Drug Administration; GEMMs, genetically engineered mouse models; HDAC, histone deacetylase; HTS, high throughput screening; NCI, National Cancer Institute; NSAID, non-steroidal anti-inflammatory drug; NF-κB, nuclear factor kappa B.

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ABSTRACT

In the current paradigm of anticancer drug development, candidate compounds are evaluated by testing their in vitro potency against molecular targets relevant to carcinogenesis; their effect on cultured cancer cells; and their ability to inhibit cancer growth in animal models. We discuss the key assumptions inherent in these approaches. In recent years, great emphasis has been placed on selecting for development compounds with nanomolar in vitro potency, expecting that they will be efficacious and safer based on the assumption that they can be used at lower doses ("*the nanomolar rule*"). However, this rule ignores critical parameters affecting efficacy and toxicity such as physiochemical and ADMET properties, off-target effects and multi-targeting activities. Thus, uncritical application of the nanomolar rule may reject efficacious compounds or select ineffective or toxic compounds. We present examples of efficacious chemotherapeutic (alkylating agents, hormonal agents, antimetabolites, thalidomide and valproic acid) and chemopreventive (aspirin and sulindac) agents having millimolar potency, and of compounds with nanomolar potency (COX-2 inhibitors) that, nevertheless, failed or proved unsafe. The effect of candidate drugs on animal models of cancer is a better predictor of human drug efficacy; particularly useful are tumor xenografts. Given the cost of failure at clinical stages, it is imperative to keep in mind the limitations of the nanomolar rule and to employ relevant in vivo models early in drug discovery to prioritize candidates. Although in vivo models will continue having a major role in cancer drug development, more robust approaches that combine high predictive ability with simplicity and low cost should be developed.

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INTRODUCTION

The treatment of cancer represents the greatest medical challenge of our times. Following the National Cancer Act of 1971 (*The War on Cancer*) introduced by President Nixon, there has been an enormous acceleration of efforts to develop effective agents against cancer. There have been notable successes, reflected in improved quality of life and cures of certain types of cancer, including some that were uniformly fatal. Yet much needs to be accomplished. Cancer still extracts a heavy toll in terms of suffering and human lives lost (Siegel et al., 2011). The need to develop additional agents remains as pressing today as ever and a worldwide effort is afoot.

During the last thirty years, pharmacology has undergone a radical transformation of its approach to drug development (Keiser et al., 2010). Whereas a generation ago a new compound was seeking a target, now, thanks to high throughput screening (HTS) and combinatorial chemistry, pharmacologists can readily identify agents affecting receptors but have to seek their potential clinical application. Nevertheless, amidst this changing landscape one critical question remains constant: *Which, if any, among several compounds that may be effective in preclinical models of cancer, will be effective in humans?*

Typically, anticancer drug development proceeds through sequential steps that involve testing of the candidate agent in cultured cancer cells, followed by testing in relevant animal tumor models. The key outcomes are efficacy and safety, with metabolism and pharmacokinetics/pharmacodynamics being important parts of this evaluation.

Varied approaches to predicting drug efficacy have been developed, none of them completely satisfactory (Suggitt and Bibby, 2005). Nevertheless, in recent years there has emerged what can be termed *the nanomolar rule*: during screening of compounds in an in vitro (reactivity towards a molecular target) or cell culture system, an agent is discarded from further analysis if its potency is not in the nanomolar range.

We believe it is time to assess the validity of this assumption. Here, we evaluate it in the context of preclinical parameters used to predict drug efficacy in its ultimate user, the human patient. We review current knowledge followed by illustrative examples that, in the absence of definitive data, provide an empirical perspective.

OVERVIEW OF PRECLINICAL SCREENING

Despite recent advances in cancer biology and the development of molecular targeted therapeutics, the attrition rate of new anticancer drugs in clinical trials is disappointingly high. It is surprising that in the age of combinatorial chemistry and HTS, novel oncology drugs undergoing clinical

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trials have shown low response rates, while offering little therapeutic advantage compared to traditional cytotoxics. Their approval rate by the Food and Drug Administration (FDA) is <5% (Hutchinson and Kirk, 2011). The failure of new drugs at the clinical stage is very costly. Thus, the predictive value of preclinical models assumes critical importance in cancer drug development.

Due to higher throughput and lower costs, in vitro screening is the current mainstay for the initial selection of drug leads. The NCI-60 screen, developed in the late 1980s, comprised 60 distinct cell lines derived from nine distinct tumor types (Shoemaker, 2006). The primary end point of the NCI-60 panel is *anti-proliferative* activity, and the profiles of cell line sensitivity may offer clues to the potential mechanisms of action using the COMPARE algorithms. In many ways, NCI-60 is tailored to the selection of coventional cytotoxic drugs. Although the NCI-60 screen identified several cytotoxic molecules, they largely act via known mechanisms. As anticancer drug discovery moves away from traditional cytotoxics to newer, molecular targeted cytostatic drugs, many emerging new anticancer entities would be considered “inactive” under the NCI-60 screen. As discussed later, many FDA-approved anticancer drugs are not nano-potent cytotoxics in vitro. Thus, this assay may have limited value in modern drug discovery, mainly due to its focus on cytotoxicity.

Currently, pharmaceutical companies use single or multiple validated targets to screen millions of potential drug leads in in vitro biochemical assays, preceding or parallel to conventional cellular screens. Inhibitors targeting protein kinases, such as imatinib and gefitinib/erlotinib, are molecular targeted drugs that are efficacious in patients whose tumors are dependent on these protein kinases. Notwithstanding the validity of the protein targets assessed, in the current paradigm of HTS, the vigorous selection of the “hit” drug leads plays a critical role in reducing attrition rates at the later stages of drug development. Molecules with nanomolar potency are highly sought after by researchers, based on the extrapolation that that such high potency predicts clinical efficacy. Such practice is widespread.

What the liberal application of the nanomolar rule seems to ignore is that in vitro potency is only one of numerous factors that contribute to the potential clinical efficacy of a given compound. Physiochemical, pharmacokinetic (absorption, distribution, metabolism and excretion (ADME)) and toxicological assessments are equally, if not more, important than the absolute potency of the agent in question. Curiously, high in vitro potency can often be gained at the expense of physiochemical properties, such as high molecular weight and increased lipophilicity (Hann, 2011). Lack of aqueous solubility, poor bioavailability and metabolic instability could reduce efficacy in humans, irrespective of a compound’s affinity to its target(s).

In vitro assays are also inadequate in the evaluation of the potential toxicity of a novel agent arising from unexpected off-target effects. Finally, many drugs have multiple targets that contribute to the overall biological response, which could not be modeled in simple HTS assays. Our drive for ultra-potent

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molecules, on the basis of in vitro assays, may come at the expense of other favorable characteristics of a drug, which contribute to the eventual failure of drug leads.

Compared to in vitro assays, in vivo tumor models are more reliable indicators of clinical efficacy in the human. Mouse tumor models are well-established means of predicting clinical outcomes (Johnson et al., 2001; Sausville and Burger, 2006). In mice, multiple parameters of drug candidates are assessed simultaneously: ADME, efficacy, and the therapeutic index□a critical parameter representing the margin between anti-tumor activity and toxicity. Here, the relevance of “nanomolar potency” is limited; a more important question is whether sufficient levels of the agent can reach its target site(s) to realize efficacy, while not causing dose-limiting side effects.

Starting in the 1950s, transplanted murine tumors (L1210 and P-388 leukemia) were used at the NCI to screen anticancer drugs, which contributed to the discovery of drugs such as vinca alkaloids and platinum salts. These murine syngeneic models are less costly and tumors grow in imunocompetent hosts; however, tumors are of mouse origin. Syngeneic tumors also have rapid growth rates, and thus have limited predictive value for the relatively slow-growing solid tumors in humans (Teicher, 2006).

With the availability of athymic *nu*/*nu* and SCID (severely combined immunodeficient) mice, human tumor subcutaneous xenografts have been extensively used in the academia and the pharmaceutical industry. The predictive value of subcutaneous human tumor xenograft models is highlighted by the retrospective analysis by NCI (Johnson et al., 2001), using data from 39 anticancer drugs whose phase II data are available. Of the anticancer drugs (33/39) that were active in one-third or more of the tested xenografts (median = 12 xenografts of various types of cancer), 45% (15/33) possessed significant clinical activity in humans. Conversely, none of the anticancer agents (6/6) with activity in less than one-third of the tested xenografts showed eventual efficacy in humans (p = 0.04). Data from the Freiburg xenograft panel are also compelling (Fiebig et al., 2004). Comparison of drug efficacy in patients and their xenografts established from biopsies revealed that patient-derived xenografts are highly predictive for responsiveness (90%, 19/21) and resistance (97%, 57/59). Such is the predictive value of patient-derived xenografts that it has been proposed that they can be employed for the optimization of treatment regimens in personalized chemotherapy (Dong et al., 2010).

The orthotopic xenograft is an alternative model where tumor cells are implanted into the organs from which they are derived; it is more physiologically relevant and is suitable for studying metastasis since subcutaneous tumors rarely metastasize. Orthotopic models, however, often require complex surgical procedures and are time consuming and costly. Nonetheless, they are useful in confirming the activity of drugs or in studying anti-metastatic therapy (Bibby, 2004). A limitation of these xenograft models is the immunosuppressed nature of the host, which precludes testing of immuno-modulatory agents and assessing the role of the immune system in the drug effect.

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Apart from predicting the clinical efficacy of treatment agents, animal models of cancer also have a major role in the discovery of chemopreventive compounds. Classical models typically involve the use of various carcinogens to induce cancer or transplanted xenografts in immunocompromised mice. Murine models of carcinogenesis readily recapitulate initiation, promotion and progression phases of neoplastic transformation, while the relevant in vitro models are few. Complementing these xenograft models are newer genetically engineered mouse models (GEMMs). GEMMs closely mimic the complex interaction of tumors with their micro-environment, an aspect of carcinogenesis missing from the xenograft models, and hosts are immunocompetent (Sharpless and Depinho, 2006). However, GEMMs are relatively costly, and there are considerable variations in the rates of tumor development and progression, which prohibit their use in large-scale screening at the present time.

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ASSUMPTIONS ASSOCIATED WITH PRECLINCIAL TESTING

As is always the case in science, in assessing results from any experimental approach, one ought to be cognizant of its limitations. Each of the three approaches to evaluate candidate drugs outlined above (drug effects on: target molecules, cultured cancer cells, animal models) includes a number of assumptions. The most critical of these assumptions are presented below.

The evaluation of the in vitro effect of a compound on a given molecule, often a protein transducing a signal vital to carcinogenesis, assumes that the in vitro effect will occur in vivo as well; that this effect will be highly specific to this particular pathway, whose inhibition is expected to kill the cancer cell; and that compounds with very low IC_{50} will have a greater likelihood to be effective in vivo and a lower possibility of side effects, since the effective drug dose will be small (assuming toxicity is proportional to dose).

Drug testing in cultured cancer cells assumes that these cells retain or largely reflect the properties of the cancer from which they originate. However, cancers even of the same type could vary considerably among individuals (Shackleton et al., 2009). Moreover, some of the properties of in situ tumors are lost during the process of their adaptation and immortalization in culture. To address these inherent limitations, investigators often resort to the evaluation of multiple cell lines (e.g. NCI-60 panel) that vary genotypically and/or phenotypically; the expectation is that if the results are consistent, they could be generalizable and thus of higher predictive value.

Animal models develop cancers either as a result of mutations (natural or engineered), exposure to carcinogens or implantation of cancer cells. Implanted cells are often of human origin and thus offer the advantage of the closest possible similarity to their intended therapeutic user. The main assumptions associated with these animal models are: ADME is similar between mice and humans; the murine tumor

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is similar to the human; and human cancers xenografted into immunocompromised animals reflect closely the human condition (even though the tumor-host interaction is missing).

A shared limitation of all three approaches is that none evaluates humans, whose genotypic and phenotypic complexity is clearly distinct from signaling molecules, cultured cells or animals. Instead, the best that preclinical testing can offer is the behavior of the candidate agent in lower animal species, most frequently the mouse.

The examples that follow (some of them summarized in Table 1) underscore the need to keep these assumptions in mind at all times. They also emphasize that the uncritical search for "nanomolar potent" drugs using simplified in vitro assays may not be optimal for the selection of drug candidates for the clinic. We review briefly effective compounds that would have been discarded during initial stages of drug discovery simply because they do not exhibit nanomolar potency and also compounds with nanomolar potency that failed during clinical testing.

EFFICACIOUS ANTICANCER AGENTS LACKING NANOMOLAR POTENCY

In this section, we describe examples of efficacious anticancer drugs that highlight the disparity between in vitro potency and in vivo efficacy.

Alkylating agents, hormonal agents and antimetabolites

Holbeck et al. (2010) surveyed in a comprehensive manner FDA-approved anticancer drugs. They used the NCI-60 panel data and grouped these clinically used drugs based on their mechanism of action, Their findings reveal that nanomolar cytotoxicity *in vitro* bears only limited relevance to clinical efficacy in humans.

Alkylating agents, among the first anticancer drugs discovered, are still the most commonly used drugs in chemotherapy. Among the 27 alkylating and DNA damaging agents evaluated, only 3 possess mean potency (GI_{50}) in the nanomolar range, while a majority (18/27) of these agents is only weakly cytotoxic (GI_{50} > 20 μM). Notably, actinomycin D, the most potent alkylating agent (GI_{50}: 1.4 nM), has limited clinical use due to its extreme toxicity (Rahman et al., 1974).

Hormonal agents are another class of chemotherapeutics highly effective in the treatment of hormone sensitive cancers (e.g. breast and prostate). However, none of 15 hormonal agents evaluated possesses nanomolar cytotoxicity. Moreover, *in vitro* activity in the NCI-60 assay did not appear to predict clinical efficacy of this group of compounds. As an example, anastrozole (GI_{50}: 2,500 μM) had significantly better clinical efficacy than tamoxifen (GI_{50}: 4.6 μM) in the ATAC trial of 9,366 postmenopausal women with estrogen receptor positive breast cancer (Howell et al., 2005).

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Similarly, most clinically effective antimetabolite drugs (14/19), such as 5-fluorouracil (GI_{50}: 18 μM), also lack nanomolar potency. The only classes of anticancer drugs with nanomolar potency in the NCI-60 panel are the tubulin-disrupting agents and topoisomerase inhibitors.

Aside from this broad survey of chemotherapeutic drugs, there are additional examples of clinically efficacious anticancer agents that lack nanomolar potency. We provide a brief overview of two such compounds, thalidomide and valproic acid.

Thalidomide

Thalidomide, a glutamic acid derivative, along with dexamethasone is a standard therapy of multiple myeloma (Fayers et al., 2011) and is under active investigation for the treatment of solid tumors (Kumar et al., 2004).

The exact mechanism(s) of thalidomide's efficacy towards cancer is not known. Thalidomide's diverse biological activities include immuno-modulatory, anti-angiogenesis and anti-inflammatory effects (Teo et al., 2005). However, thalidomide is inactive in the NCI-60 screen (GI_{50} >100 μM) (Holbeck et al.) and it only slightly reduces the proliferation of multiple myeloma cells (<20%) at high concentrations (>100 μM) (Hideshima et al., 2000). Moreover, thalidomide did not significantly inhibit angiogenesis in vitro in the absence of a microsomal preparation (Dredge et al., 2002). On the contrary, thalidomide significantly inhibited tumor growth in mice bearing xenografts derived from multiple myeloma (Yaccoby et al., 2002; Lentzsch et al., 2003), esophageal squamous cell carcinoma, lung cancer, cervical cancer and melanoma, consistently demonstrating strong inhibition of angiogenesis in vivo (Table 1). Its efficacy is further enhanced when combined with chemotherapy. The combination of bevacizumab, thalidomide and docetaxel was highly effective (>70 %) in inhibiting prostate xenografts in mice; and such a combination of anti-angiogenic and cytotoxic agents also showed remarkable efficacy in a phase II trial in patients with metastatic prostate cancer (Ning et al., 2010).

Thalidomide has considerable clinical efficacy despite an apparent lack of in vitro potency. In vivo, thalidomide undergoes spontaneous degradation as well as enzymatic metabolism, resulting in over 20 metabolites. Part of the biological activity of thalidomide, such as inhibition of angiogenesis, requires metabolic activation in vivo by cytochrome P450 2C19 (Bauer et al., 1998). Perhaps more important is the multifaceted nature of the biological activities of thalidomide, involving multiple aspects of malignant cell growth, which may act synergistically in vivo to produce greater efficacy, as in the case of multiple myeloma (Franks et al., 2004).

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Valproic acid

Valproic acid (VPA), a branched short-chain fatty acid, is widely used as an antiepileptic drug. Recently, it emerged that VPA has promising anticancer properties. VPA is an inhibitor of histone deacetylase (HDAC) (Gottlicher et al., 2001). Phase I/II trials with VPA, alone or in combination with chemotherapy, have shown clinical efficacy towards various malignancies (Table 1).

In vitro, VPA is a weak inhibitor of HDAC. It inhibits the activities of HDAC isoforms in high micromolar to low millimolar range (0.4-2 mM). Moreover, VPA has low cytotoxic activity against cancer cell lines with IC_{50} values between 0.5-6 mM. Thus, it is unlikely that VPA would emerge as a drug lead using the nanomolar rule. In contrast, VPA has shown potent antitumor activity in animal models. VPA strongly suppresses the growth of cancer xenografts (Table 1). Study in breast xenograft models revealed a potential synergism between VPA and epirubicin (Marchion et al., 2005), a combination which subsequently showed promising efficacy in phase I/II trials (Munster et al., 2007; Munster et al., 2009).

A critical evaluation of the disparate in vitro and in vivo activity of VPA can be appropriately implemented in the context of its pharmacokinetic and toxicity profiles. VPA possesses a high oral bioavailability and is well tolerated by patients. VPA up to 75-120 mg/kg/day was well tolerated (Munster et al., 2007; Daud et al., 2009; Munster et al., 2009), resulting in plasma levels in the range of 1.2-1.5 mM. Correspondingly, there is clinical inhibition of HDACs in peripheral-blood mononuclear cells (Munster et al., 2007; Daud et al., 2009) and in tumor tissues (Munster et al., 2009).

CHEMOPREVENTIVE AGENTS LACKING NANOMOLAR POTENCY

The recent definitive documentation that aspirin is a chemopreventive agent against colon cancer has energized this nascent field, providing the impetus to develop effective and safe chemopreventive agents. Cancer chemoprevention, in many ways akin to the pharmacological prevention of cardiovascular diseases, is preferable to chemotherapy as it minimizes human suffering, saves lives and reduces health care costs.

From a drug development point of view, chemopreventive agents present different challenges compared to chemotherapeutic agents. Importantly, chemopreventive agents target tumor biology at an earlier stage, when the complexity of the neoplastic cell is lower compared to full-blown cancer requiring chemotherapy. On the other hand, agent safety is far more critical: chemoprevention agents are administered to otherwise healthy subjects for the rest of their lives, whereas chemotherapeutic agents are given to a patient at imminent risk to his life, when less safe agents are acceptable. Thus, in selecting chemopreventive agents, animal data that incorporate ADME and safety parameters generate highly consequential information.

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Below, we discuss two prototypical chemopreventive agents, aspirin and sulindac; both would have been discarded if the nanomolar rule had been applied to them.

Aspirin

Epidemiological evidence has suggested that long-term aspirin use reduces the risk of colorectal neoplasia (Arber, 2000; Elwood et al., 2009). Randomized clinical trials showed that low-dose aspirin prevented the development of polyps (Baron et al., 2003), while high-dose aspirin suppressed recurrent adenomas in patients who previously had colorectal cancer (Sandler et al., 2003).

Aspirin and salicylic acid, its major metabolite, exhibit very weak cytotoxicity towards cancer cell lines (IC_{50} = 2-5 mM). Aspirin is only a weak inhibitor of COX-2, the COX isoform implicated in carcinogenesis. Nonetheless, aspirin is effective in animal models of cancer, including chemically-induced lung and colon cancer, human cancer xenografts, as well as *Apc*^{Min} mice (Table 1).

Despite poor in vitro potency, aspirin has shown considerable efficacy in humans. Similar to valproic acid, the activity of aspirin should be evaluated in the context of its pharmacokinetic properties, which can be assessed comprehensively only in vivo. Plasma salicylate levels can reach up to 1 mM with high dose aspirin (325 mg), which is sufficient to inhibit the in vivo production of prostaglandin E_2, a tumor promoter. Clinical trials with aspirin are ongoing, due to the mounting evidence that aspirin can reduce the risk of various cancers.

Sulindac

Sulindac is a nonsteroidal anti-inflammatory drug (NSAID) used for the treatment of arthritis. Early clinical trials found that sulindac regressed polyps in patients with familial adenomatous polyposis (FAP) (Labayle et al., 1994). A recent clinical trial showed that a combination of sulindac and difluoromethylornithine reduced recurrence of adenomas by 70% and advanced adenomas by 95% (Meyskens et al., 2008).

Sulindac inhibits carcinogenesis through cyclooxygenase (COX)-dependent and -independent mechanisms (Kashfi and Rigas, 2005). Sulindac is a prodrug which is reduced to sulindac sulfide, a COX inhibitor, or oxidized to sulindac sulfone. Neither of these compounds has potent cytotoxicity *in vitro*. Sulindac sulfide is the most potent, with IC_{50} values ~100 µM, while sulindac and sulindac sulfone are weakly cytotoxic, with IC_{50} values between 0.3-5 mM. The binding affinities of sulindac and its metabolites to their targets are also moderate. On the basis of in vitro data, the anticancer potential of sulindac appears unimpressive. In vivo models, however, suggest another story. Sulindac was highly active (>75% inhibition) in arresting the growth of human colon, prostrate, head and neck cancer xenografts (Table 1). Sulindac also significantly arrested tumor growth in *Apc*^{Min} mice, a model of FAP. Synergy between sulindac and difluoromethylornithine was also reproduced in *Apc*^{Min} mice (Ignatenko et al., 2008). The

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lack of satisfactory in vitro□in vivo correlation of the antitumor activity of sulindac may be attributed to the multi-targeted nature of this drug.

CLINICALLY FAILED COMPOUNDS WITH NANOMOLAR POTENCY

The widely publicized withdrawal of COX-2 specific inhibitors following their clinical testing for cancer provides an instructive example of compounds with remarkably high (nanomolar) potency in vitro that proved neither efficacious nor safe to be clinically useful.

The observations that COX-2 is largely responsible for production of prostaglandins in inflammation and cancer, in which it is over-expressed (Fournier and Gordon, 2000), have led to the notion that specific inhibition of COX-2 could prevent or treat cancer. The COX-2 selective inhibitors rofecoxib, celecoxib and valdecoxib inhibited COX-2 activity with nanomolar potency (IC_{50}: 5-500 nM), while being less active towards COX-1 (Gierse et al., 2005). It was hoped that, compared to NSAIDs, they would offer higher efficacy and improved gastrointestinal safety since they spared COX-1 (Warner et al., 1999). Coxibs were assessed for both their chemopreventive and chemotherapeutic applications to cancer. However, they failed to meet the dual expectation of efficacy and lower toxicity in clinical trials for both applications.

Treatment of FAP patients with celecoxib for 6 months reduced the number of colorectal polyps by 28% (Steinbach et al., 2000); rofecoxib had a marginal effect (6.8% reduction) (Higuchi et al., 2003). Rofecoxib was also ineffective in improving overall survival in the adjuvant setting of colorectal cancer (Midgley et al., 2010). These results are in stark contrast to the high efficacy of sulindac. Other disappointing results were the recently reported clinical failures of celecoxib and rofecoxib to provide clinical benefits when combined with chemotherapy/EGFR inhibitors in treating non-small cell lung cancer (Gridelli et al., 2007; O'Byrne et al., 2007; Edelman et al., 2008; Fidler et al., 2008). Consistent with these findings, celecoxib only has a moderate inhibitory effect in human colon xenograft models, whereas neither rofecoxib nor etoricoxib possess significant antitumor activity in vivo (Schiffmann et al., 2008).

The chemopreventive efficacy of rofecoxib was also evaluated in the Adenomatous Polyp Prevention on VIOXX (APPROVe) trial (Baron et al., 2006). Rofecoxib treatment was associated with a modest reduction (24 %) in the risk of colorectal adenomas. However, it caused a 1.92-fold increase in serious thrombotic cardiovascular events (Bresalier et al., 2005). This led to the high profile withdrawal of rofecoxib and valdecoxib from the market and limitations in the clinical use of celecoxib. The Achilles heel of the coxibs lies in their potency towards COX-2, leading to selective suppression of the COX-2-dependent cardioprotective prostacyclin (Fries and Grosser, 2005). Chronic intake of coxibs therefore heightens the risk of thrombosis. The limited efficacy and adverse cardiovascular effects of coxibs have cast major doubts on the feasibility of COX-2 inhibition as a strategy for cancer prevention and treatment.

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Mechanistic work on coxibs underscores another limitation of the nanomolar rule. There is now evidence that targets other than COX-2 contribute to the anticancer effects of celecoxib, such as NF-κB, phosphodiesterases and MAPKs (Kashfi and Rigas, 2005).

CONCLUSIONS

Prediction of anticancer efficacy using preclinical models remains difficult and requires further development. Investigators evaluating candidate drugs should be constantly aware of the limitations of preclinical approaches. In recent years, potency in in vitro assays has become a predominant influence on screening and selection of drug leads. While nanomolar potency remains desirable, it has no predictive value in complex diseases such as cancer. We believe that a more holistic approach that strives to achieve a judicious balance between potency, solubility, bioavailability, metabolic stability and safety of candidate drugs, would facilitate the discovery of novel therapeutics for cancer.

Key to this multi-parameter evaluation is the use of appropriate in vivo models. In vivo models, such as subcutaneous and orthotopic xenografts in mice, when properly used, are more reliable indicators of clinical efficacy. Indeed, none of the clinically approved agents has lacked activity in the mouse xenograft models. Patient-derived xenografts are highly predictive of drug response, and have great potential in individualized medicine. Until more reliable approaches are developed, we foresee that due to their predictive capabilities, in vivo models, imperfect as they are, will continue to play a major role in the development of novel drugs. Every effort should be made to develop new approaches that combine high predictive ability with simplicity and low cost.

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REFERENCES

Arber N (2000) Do NSAIDs prevent colorectal cancer? *Can J Gastroenterol* **14**:299-307.

Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, McKeown-Eyssen G, Summers RW, Rothstein R, Burke CA, Snover DC, Church TR, Allen JI, Beach M, Beck GJ, Bond JH, Byers T, Greenberg ER, Mandel JS, Marcon N, Mott LA, Pearson L, Saibil F and van Stolk RU (2003) A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* **348**:891-899.

Baron JA, Sandler RS, Bresalier RS, Quan H, Riddell R, Lanas A, Bolognese JA, Oxenius B, Horgan K, Loftus S and Morton DG (2006) A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. *Gastroenterology* **131**:1674-1682.

Bauer KS, Dixon SC and Figg WD (1998) Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. *Biochem Pharmacol* **55**:1827-1834.

Bibby MC (2004) Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* **40**:852-857.

Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA and Baron JA (2005) Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* **352**:1092-1102.

Daud AI, Dawson J, DeConti RC, Bicaku E, Marchion D, Bastien S, Hausheer FA, 3rd, Lush R, Neuger A, Sullivan DM and Munster PN (2009) Potentiation of a topoisomerase I inhibitor, karenitecin, by the histone deacetylase inhibitor valproic acid in melanoma: translational and phase I/II clinical trial. *Clin Cancer Res* **15**:2479-2487.

Dong X, Guan J, English JC, Flint J, Yee J, Evans K, Murray N, Macaulay C, Ng RT, Gout PW, Lam WL, Laskin J, Ling V, Lam S and Wang Y (2010) Patient-derived first generation xenografts of non-small cell lung cancers: promising tools for predicting drug responses for personalized chemotherapy. *Clin Cancer Res* **16**:1442-1451.

Dredge K, Marriott JB, Macdonald CD, Man HW, Chen R, Muller GW, Stirling D and Dalgleish AG (2002) Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects. *Br J Cancer* **87**:1166-1172.

Edelman MJ, Watson D, Wang X, Morrison C, Kratzke RA, Jewell S, Hodgson L, Mauer AM, Gajra A, Masters GA, Bedor M, Vokes EE and Green MJ (2008) Eicosanoid modulation in advanced lung cancer: cyclooxygenase-2 expression is a positive predictive factor for celecoxib + chemotherapy--Cancer and Leukemia Group B Trial 30203. *J Clin Oncol* **26**:848-855.

Elwood PC, Gallagher AM, Duthie GG, Mur LA and Morgan G (2009) Aspirin, salicylates, and cancer. *Lancet* **373**:1301-1309.

Fayers PM, Palumbo A, Hulin C, Waage A, Wijermans P, Beksac M, Bringhen S, Mary JY, Gimsing P, Termorshuizen F, Haznedar R, Caravita T, Moreau P, Turesson I, Musto P, Benboubker L, Schaafsma M, Sonneveld P and Facon T (2011) Thalidomide for previously untreated elderly

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JPET Fast Forward. Published on March 23, 2012 as DOI: 10.1124/jpet.112.191957
This article has not been copyedited and formatted. The final version may differ from this version.

patients with multiple myeloma: meta-analysis of 1685 individual patient data from 6 randomized clinical trials. *Blood* **118**:1239-1247.

Fidler MJ, Argiris A, Patel JD, Johnson DH, Sandler A, Villaflor VM, Coon Jt, Buckingham L, Kaiser K, Basu S and Bonomi P (2008) The potential predictive value of cyclooxygenase-2 expression and increased risk of gastrointestinal hemorrhage in advanced non-small cell lung cancer patients treated with erlotinib and celecoxib. *Clin Cancer Res* **14**:2088-2094.

Fiebig HH, Maier A and Burger AM (2004) 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* **40**:802-820.

Fournier DB and Gordon GB (2000) COX-2 and colon cancer: potential targets for chemoprevention. *J Cell Biochem Suppl* **34**:97-102.

Franks ME, Macpherson GR and Figg WD (2004) Thalidomide. *Lancet* **363**:1802-1811.

Fries S and Grosser T (2005) The cardiovascular pharmacology of COX-2 inhibition. *Hematology Am Soc Hematol Educ Program*:445-451.

Gierse JK, Zhang Y, Hood WF, Walker MC, Trigg JS, Maziasz TJ, Koboldt CM, Muhammad JL, Zweifel BS, Masferrer JL, Isakson PC and Seibert K (2005) Valdecoxib: Assessment of cyclooxygenase-2 potency and selectivity. *J Pharmacol Exp Ther* **312**:1206-1212.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG and Heinzel T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* **20**:6969-6978.

Gridelli C, Gallo C, Ceribelli A, Gebbia V, Gamucci T, Ciardiello F, Carozza F, Favaretto A, Daniele B, Galetta D, Barbera S, Rosetti F, Rossi A, Maione P, Cognetti F, Testa A, Di Maio M, Morabito A and Perrone F (2007) Factorial phase III randomised trial of rofecoxib and prolonged constant infusion of gemcitabine in advanced non-small-cell lung cancer: the GEmcitabine-COxib in NSCLC (GECO) study. *Lancet Oncol* **8**:500-512.

Hann MM (2011) Molecular obesity, potency and other addictions in drug discovery. *Medchemcomm* **2**:349-355.

Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai YT, Treon SP, Lin B, Schlossman RL, Richardson P, Muller G, Stirling DI and Anderson KC (2000) Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* **96**:2943-2950.

Higuchi T, Iwama T, Yoshinaga K, Toyooka M, Taketo MM and Sugihara K (2003) A randomized, double-blind, placebo-controlled trial of the effects of rofecoxib, a selective cyclooxygenase-2 inhibitor, on rectal polyps in familial adenomatous polyposis patients. *Clin Cancer Res* **9**:4756-4760.

Holbeck SL, Collins JM and Doroshow JH (2010) Analysis of Food and Drug Administration-approved anticancer agents in the NCI60 panel of human tumor cell lines. *Mol Cancer Ther* **9**:1451-1460.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 23, 2012 as DOI: 10.1124/jpet.112.191957
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Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, Hoctin-Boes G, Houghton J, Locker GY and Tobias JS (2005) Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* **365**:60-62.

Hutchinson L and Kirk R (2011) High drug attrition rates--where are we going wrong? *Nat Rev Clin Oncol* **8**:189-190.

Ignatenko NA, Besselsen DG, Stringer DE, Blohm-Mangone KA, Cui H and Gerner EW (2008) Combination chemoprevention of intestinal carcinogenesis in a murine model of familial adenomatous polyposis. *Nutr Cancer* **60 Suppl 1**:30-35.

Johnson JI, Decker S, Zaharevitz D, Rubinstein LV, Venditti JM, Schepartz S, Kalyandrug S, Christian M, Arbuck S, Hollingshead M and Sausville EA (2001) Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br J Cancer* **84**:1424-1431.

Kashfi K and Rigas B (2005) Non-COX-2 targets and cancer: expanding the molecular target repertoire of chemoprevention. *Biochem Pharmacol* **70**:969-986.

Keiser MJ, Irwin JJ and Shoichet BK (2010) The chemical basis of pharmacology. *Biochemistry* **49**:10267-10276.

Kumar S, Witzig TE and Rajkumar SV (2004) Thalidomid: current role in the treatment of non-plasma cell malignancies. *J Clin Oncol* **22**:2477-2488.

Labayle D, Boyer J, Drouhin F, Zarka Y and Fischer D (1994) Sulindac in familial adenomatous polyposis. *Lancet* **343**:417-418.

Lentzsch S, LeBlanc R, Podar K, Davies F, Lin B, Hideshima T, Catley L, Stirling DI and Anderson KC (2003) Immunomodulatory analogs of thalidomide inhibit growth of Hs Sultan cells and angiogenesis in vivo. *Leukemia* **17**:41-44.

Marchion DC, Bicaku E, Daud AI, Sullivan DM and Munster PN (2005) Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. *Cancer Res* **65**:3815-3822.

Meyskens FL, Jr., McLaren CE, Pelot D, Fujikawa-Brooks S, Carpenter PM, Hawk E, Kelloff G, Lawson MJ, Kidao J, McCracken J, Albers CG, Ahnen DJ, Turgeon DK, Goldschmid S, Lance P, Hagedorn CH, Gillen DL and Gerner EW (2008) Difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas: a randomized placebo-controlled, double-blind trial. *Cancer Prev Res (Phila)* **1**:32-38.

Midgley RS, McConkey CC, Johnstone EC, Dunn JA, Smith JL, Grumett SA, Julier P, Iveson C, Yanagisawa Y, Warren B, Langman MJ and Kerr DJ (2010) Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. *J Clin Oncol* **28**:4575-4580.

Munster P, Marchion D, Bicaku E, Lacevic M, Kim J, Centeno B, Daud A, Neuger A, Minton S and Sullivan D (2009) Clinical and biological effects of valproic acid as a histone deacetylase inhibitor

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JPET Fast Forward. Published on March 23, 2012 as DOI: 10.1124/jpet.112.191957
This article has not been copyedited and formatted. The final version may differ from this version.

on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clin Cancer Res* **15**:2488-2496.

Munster P, Marchion D, Bicaku E, Schmitt M, Lee JH, DeConti R, Simon G, Fishman M, Minton S, Garrett C, Chiappori A, Lush R, Sullivan D and Daud A (2007) Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. *J Clin Oncol* **25**:1979-1985.

Ning YM, Gulley JL, Arlen PM, Woo S, Steinberg SM, Wright JJ, Parnes HL, Trepel JB, Lee MJ, Kim YS, Sun H, Madan RA, Latham L, Jones E, Chen CC, Figg WD and Dahut WL Phase II trial of bevacizumab, thalidomide, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol* **28**:2070-2076.

O'Byrne KJ, Danson S, Dunlop D, Botwood N, Taguchi F, Carbone D and Ranson M (2007) Combination therapy with gefitinib and rofecoxib in patients with platinum-pretreated relapsed non small-cell lung cancer. *J Clin Oncol* **25**:3266-3273.

Rahman YE, Cerny EA, Tollaksen SL, Wright BJ, Nance SL and Thomson JF (1974) Liposome-encapsulated actinomycin D: potential in cancer chemotherapy. *Proc Soc Exp Biol Med* **146**:1173-1176.

Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, Petrelli N, Pipas JM, Karp DD, Loprinzi CL, Steinbach G and Schilsky R (2003) A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* **348**:883-890.

Sausville EA and Burger AM (2006) Contributions of human tumor xenografts to anticancer drug development. *Cancer Res* **66**:3351-3354.

Schiffmann S, Maier TJ, Wobst I, Janssen A, Corban-Wilhelm H, Angioni C, Geisslinger G and Grosch S (2008) The anti-proliferative potency of celecoxib is not a class effect of coxibs. *Biochem Pharmacol* **76**:179-187.

Shackleton M, Quintana E, Fearon ER and Morrison SJ (2009) Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell* **138**:822-829.

Sharpless NE and Depinho RA (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* **5**:741-754.

Shoemaker RH (2006) The NCI60 human tumour cell line anticancer drug screen. *Nat Rev Cancer* **6**:813-823.

Siegel R, Ward E, Brawley O and Jemal A (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* **61**:212-236.

Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK and Levin B (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* **342**:1946-1952.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 23, 2012 as DOI: 10.1124/jpet.112.191957
This article has not been copyedited and formatted. The final version may differ from this version.

Suggitt M and Bibby MC (2005) 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. *Clin Cancer Res* **11**:971-981.

Teicher BA (2006) Tumor models for efficacy determination. *Mol Cancer Ther* **5**:2435-2443.

Teo SK, Stirling DI and Zeldis JB (2005) Thalidomide as a novel therapeutic agent: new uses for an old product. *Drug Discov Today* **10**:107-114.

Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA and Vane JR (1999) Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc Natl Acad Sci U S A* **96**:7563-7568.

Yaccoby S, Johnson CL, Mahaffey SC, Wezeman MJ, Barlogie B and Epstein J (2002) Antimyeloma efficacy of thalidomide in the SCID-hu model. *Blood* **100**:4162-4168.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 23, 2012 as DOI: 10.1124/jpet.112.191957
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FOOTNOTES:

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Table 1. The in vitro effects and in vivo efficacy of anticancer agents

Drug	In vitro IC_{50}	Affinity to putative target	Animal model efficacy	Human efficacy
Thalidomide	> 100 μM [1]	TNFα inhibition (0.2 mM) [2]	Multiple myeloma [3], colon cancer [4, 5], cervical cancer [6], squamous cell carcinoma [7], lung cancer [8, 9], melanoma [10], prostate cancer [11]	Multiple myeloma [12-30],colorectal cancer [31], lung cancer [32], prostate cancer [11, 33], renal cancer [34], Kaposi's sarcoma [35, 36]
Valproic acid	0.5-5 mM [37-41]	HDACs (0.5 to 2 mM) [42]	Neuroblastoma [43]; prostate cancer [44-47], cervical cancer [48], NSCLC [49] , sarcoma rhabdomyosarcoma [50], melanoma [51], osteosarcoma [52], colon cancer [53]	Leukemia [54-56], breast cancer [57, 58], prostate cancer, pancreatic cancer [59] and neurological cancers [60, 61]
Sulindac	0.6- 1 mM [62-67]	COX-1: >100 μM COX-2: >100 μM [68]	Colon cancer [69, 70], prostate cancer [71], , NSCLC [72], head and neck cancer [73], uterine cancer [74], pancreatic cancer [75], colon cancer	Familial adenomatous polyposis [76-80], combination with DFMO reduced recurrence of adenomas by 70% and of more advanced adenomas by 95% [81]
Aspirin	2-5 mM [82-84]	COX-1: 2.3 μM COX-2: 3.6 μM [85]	Colon cancer [86], fibrosarcoma [87], lung cancer [88-90], cervical cancer [91]	Colorectal polyp recurrence [92, 93].
Rofecoxib	-	COX-2: >1 mM COX-2: 500 nM [94]	Gastric cancer [95], lung cancer [96]	Marginal efficacy in colon polyps, withdrawn due to adverse cardiovascular effects
Celecoxib	10-75 μM [97-101]	COX-1: 15 μM COX-2: 40 nM [94]	Colon cancer [97, 101, 102], liver cancer [98, 103], bladder cancer [98], prostate cancer [99], neuroblastoma [100], breast cancer [104]	Colorectal polyp recurrence, but significantly raises the risk of serious cardiovascular events [105].

The references for this table are listed in the supplementary information.

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