Cellular pharmacology studies of anticancer agents: recommendations from the EORTC-PAMM group

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Abstract

An increasing number of manuscripts focus on the *in vitro* evaluation of established and novel antitumor agents in experimental models. Whilst the design of such *in vitro* assays is inherently flexible, some of these studies lack the minimum information necessary to critically evaluate their relevance or have been carried out under unsuitable conditions. The use of appropriate and robust methods and experimental design has important implications for generating results that are reliable, relevant and reproducible. The Pharmacology and Molecular Mechanisms (PAMM) group of the European Organization for Research and Treatment of Cancer (EORTC) is the largest group of academic scientists working on drug development and bundles decades of expertise in this field. This position paper addresses all researchers with an interest in the preclinical and cellular pharmacology of antitumor agents and aims at generating basic recommendations for the correct use of compounds to be tested for antitumor activity by using a range of preclinical cellular models of cancer.

Keywords: cellular pharmacology, anticancer agents, guidelines

Introduction

Pharmacology is a complex multidisciplinary science that acts in concert with sister sciences including medicinal chemistry, cell biology and physiology, medicine and pharmaceutics aiming at identifying useful agents for disease treatment. Over the years the interest of researchers in pharmacology, particularly those working at the preclinical level, has increased dramatically in several fields including research against cancer. In contrast, in the past few decades researchers at several start-up companies, in particular small biotech, and molecular biologists neglected pharmacology in drug development and had to learn the hard way the importance of insight in drug pharmacology. A key player in the preclinical evaluation of novel compounds and established anticancer drugs is the use of cellular models of cancer for chemosensitivity studies. This methodology plays a fundamental role in experimental oncology studies designed to (i) identify new potential therapeutic agents, (ii) determine the mechanism of action (MOA) and (iii) understand pharmacological factors that control the cellular response. The design of *in vitro* tumor cell sensitivity assays is inherently simple and flexible allowing a wide range of variables to be tested. However, this simplicity and flexibility is a "double-edged sword" and may generate results that cannot always be accurately interpreted or easily reproduced in other laboratories. There are many examples of cases where basic errors are made in experimental design that can lead to misleading or even erroneous results. Furthermore, basic researchers sometimes produce results that have little real pharmacological relevance, a consequence of using drug exposure conditions that cannot be achieved in an organism [1-3]. In fact, there are examples from the literature showing that different mechanisms are activated in response to drug treatment in cell lines depending on the drug concentrations [4].

Applying proper test conditions was a joint collaborative effort of several EORTC groups, the Pharmacology and Molecular Mechanisms Groups (PAMM), Screening and Pharmacology Group (SGP), New Drug Development group, the Cancer Research Campaign (CRC, now

CRUK) and the Development Therapeutics Program (DTP) of the National Cancer Institute (NCI) in Bethesda, MD, USA [3]. In this joint program, the so-called NCI-compounds group, a workflow was developed to test new compounds coming from the NCI-60 cell line program of the NCI, which was based on using proper pharmacology tools to speed up selection of potentially active new chemical entities (NCE).The purpose of this article is to provide a series of suggestions to ensure that scientific integrity in the conduct of *in vitro* chemosensitivity testing using cellular models is maintained at the highest possible standards. This paper will effectively be segregated into two key areas of activity (i) *in vitro* evaluation of anticancer drugs that are in clinical trial or are approved for use in humans and (ii) novel compounds that are entering preclinical testing for the first time. In addition, this manuscript will also discuss some of the experimental models that are being used to evaluate novel and established anticancer agents.

This article presents the views of members of the EORC-PAMM group. PAMM comprises researchers working in the fields of pharmacology, pharmacokinetics-pharmacodynamics, pharmacogenetics-pharmacogenomics, molecular mechanisms of anticancer drug effects and drug-related molecular pathology (http://www.eortc.org/research-field/pharmacology-molecular-mechanisms). Our views are designed to present general criteria for preclinical studies in which compounds with potential anticancer activity or well-known antitumor agents are tested, whilst respecting the fact that the design of *in vitro* tumor cell sensitivity testing is inherently flexible.

Design of *in vitro* tumor cell sensitivity assay: general principles

The basic design of *in vitro* tests for evaluation of tumor cell sensitivity is simple and is illustrated in Figure 1. For adherent cell lines, cells are plated into cell culture dishes and allowed to ‘adhere and adapt’ to the culture environment for a period of time (typically

overnight or 24 hours). Following this time period, cells are treated with the therapeutic agent in question for either (i) a defined, short duration of exposure followed by a recovery period to allow cells to respond or (ii) continuous drug exposure. Following drug exposure to the test agent, cellular response can be assessed by a variety of endpoints and these can be broadly divided into (i) clonogenic or (ii) non-clonogenic endpoints [5-7]. Collectively, such tests can be referred to as chemosensitivity assays, although originally the prefix "chemo" denoted chemical agents that kill microbes or tumor cells. However, in real life, similar assays can be employed for multiple tests including chemotherapeutic agents and radiotherapy [8]. It should be noted that in this paper "chemotherapy" indicates any chemical entity foreign to the human body, including the "classical" cytotoxic compounds (e.g. antimetabolites, nitrogen mustards, platinum compounds, tubulin antagonists, anti-tumor antibiotics, etc), as well as novel so-called targeted drugs, such as tyrosine kinase inhibitors (TKI), anti-angiogenesis drugs, proteasome inhibitors, etc. Each endpoint has its own advantage and disadvantage and the choice of which one to use is generally based on individual researcher's preferences or specific objectives (measuring growth inhibition, cell kill, cell survival or effects on cell population growth, for example). For suspension cultures, the procedure is effectively the same except for the initial conditioning phase which is not mandatory. It is important to recognize that not one single assay should be performed as a stand-alone test from which to draw firm conclusions regarding *in vitro* activity against cells.

Whilst the basic design of *in vitro* assays is straightforward, the simplicity of this approach obscures a multitude of factors that can influence cellular response and therefore affect the quality and interpretation of the data obtained. A good understanding of these factors is essential to the design of all *in vitro* chemosensitivity experiments, but is particularly so for those addressing specific aspects of tumor biology and drug pharmacology. The following sections highlight some of these issues in the context of enhancing good experimental design and avoiding poor practice.

(i) The use of pharmacologically relevant drug concentrations for established drugs

Two key pharmacological parameters that determine cellular response are the concentration (C) of a drug and the duration of drug exposure (T). For the majority of cytotoxic drugs, cellular response is usually proportional to the product of C x T with the exception of drugs that are cell cycle phase specific, where cellular response above a certain threshold C is typically proportional to T. In this case, extending the duration of drug exposure allows more cells to enter the sensitive phase of the cell cycle. For drugs where pharmacokinetics data are available (i.e., those that have been approved for use in humans or are in advanced preclinical or early clinical trial), it is essential that drug exposure conditions do not exceed those that are pharmacologically achievable either in humans. In general terms, therefore, the selection of exposure parameters for use *in vitro* should not exceed the total plasma exposure parameters (i.e., area under the curve) achieved *in vivo*, and the concentrations in preclinical cell-based models should aim to be in the same range as the plasma concentrations achievable *in vivo* (Figure 2). This rule is best applicable to haematological malignancies where the target organ is the blood itself, whereas less is known about the C x T parameters achieved in solid tumors.

A series of examples where the effects of using drug exposure parameters that are not pharmacologically relevant is given below. Concentrations of anticancer drugs in patients vary over several orders of magnitude. The choice of adequate concentrations for *in vitro* experiments is critical, because killing cancer cells can be achieved with almost every compound if the concentration is high enough. Results of preclinical experiments using very high concentrations can be misleading and often result in false conclusions. For example, as a result of using non-pharmacologically relevant concentrations in such experiments, there is great confusion about the mechanism of action of anthracyclines in the literature. As pointed out by Gerwitz [9] almost 20 years ago, many of the proposed mechanisms for both cytotoxicity and cardiotoxicity of anthracyclines are not relevant in patients because of exorbitant high drug concentrations used in the preclinical experiments, even exceeding the

high peak plasma concentrations achieved during short-term infusion of anthracyclines. Nevertheless, mechanisms such as free radical formation for the anti-tumor effects of anthracyclines are still mentioned in many textbooks. To complicate the scenario about the concentrations of anthracyclines in the tumor, a high and variable tumor to plasma concentration ratio of doxorubicin was found in breast cancer patients [10]. Indeed, a high tumour to plasma concentration ratio is a result of enrichment of the drug within the tumour cells, not in the interstitium, and this is the case for doxorubicin.

Drug penetration barriers (consisting of influx and efflux pumps) exist for a number of anticancer drugs and this - combined with the effect of high interstitial fluid pressure on drug penetration into tumors - significantly modifies the C x T parameters experienced by tumor cells [11]. The presence of a poor and inefficient vascular supply to tumors leads to the establishment of a hypoxic microenvironment where drug delivery is significantly impaired [12]. It is technically feasible to measure drug concentrations within a tumor but practically, this was rarely done [13], but is getting more common, both for standard cytotoxic and novel TKI therapy. In fact, in many current clinical protocols, taking tumor biopsy specimens is mandatory and often drug concentrations and other parameters are measured [14]. Basically, several methods are available to measure drug concentrations in tissues and include (i) quantification in homogenized tumor tissue after surgical excision; (ii) microdialysis by inserting a microdialysis needle placed directly into the tumor; (iii) the use of radioactively labelled drugs (e.g., short-lived radiolabels, such as ^{18}F); (iv), the use of imaging techniques such as NMR and more recently mass-spectrometry based assays. All methods have many drawbacks limiting their applicability. Therefore, the plasma concentration has remained the golden standard because it has been established for most drugs. Plasma concentrations certainly do not give any information on intra-tumoral heterogeneity in drug distribution, and do not give any information of drug sequestration, both in the tumor and in normal tissues. For instance, a high lysosomal accumulation of sunitinib was found in tumors, due to sequestration in

lysosomes [15, 16]. Since lysosomes are less abundant in normal cells, the total cellular accumulation is less. Also for the classical anticancer drug 5-fluorouracil (5FU) high concentrations (1-10 pmol/mg tumor tissue) have been reported in the tumor, even days after administration, in contrast to the rapid plasma half-life with sub nM concentrations after 2 days [17]. The latter is due to sequestration in polar metabolites and high-molecular weight molecules such as RNA. It should also be recognized that many of the novel drugs (e.g., all TKI) are > 95% protein bound [18], or due to their physico-chemical properties are trapped in tissues. In animal models, it is usually easier to determine drug concentrations (and their retention) in the target tissues (tumor and normal tissues) and get a better indication of the C x T. With novel tools such as Positron Emission Tomography (PET), it is currently possible to determine the drug concentration in several tissues [19, 20]. In several studies, serum concentrations are reported. It should be noted that serum and plasma are often exchanged, while serum concentrations are sometimes higher than plasma, since some drugs tend to accumulate in platelets, which are lysed during serum preparation. A relative easy alternative for drug trapping is the measurement of drug accumulation in red blood cells, in which drugs such as the above-mentioned sunitinib and 5FU tend to be retained longer than in plasma. Despite this, plasma concentrations after administering therapeutic drug concentrations in humans still remain a suitable guide for the selection of conditions for preclinical experiments to ensure the most relevant parameters to be used *in vitro*.

In the absence of accurate information on the concentration of anticancer drugs in tumors, investigators should be encouraged to study putative mechanisms of action of drugs on cultured cells at concentrations that impact a therapeutic response (e.g., concentrations that inhibit cell growth/survival by 50-90%, IC_{50} - IC_{90}, Figure 2). The number of cell lines used for these studies could vary depending on the molecular hetetogeneity of the tumor type as well as on rarity or frequence of the disease. IC_{50} values are sometimes reported as GI_{50} (test agent concentration which inhibits growth by 50%; e.g. in data from the NCI-60 panel), while TGI

represents total growth inhibition and LC_{50} ("lethal concentration", concentration leading to death of 50% initially seeded cells – or its equivalent) response parameters (see for detailed definitions: https://dtp.cancer.gov/discovery_development/nci-60/methodology.htlm). By including a simple end-point (surrogate for cell count) at the time of test agent addition, one can easily ascertain cell growth, test agent concentrations which inhibit net cell growth and concentrations which cause net cell death [21, 22]. Thus, one can begin to distinguish cytotoxic agents/agent concentrations from cytostatic ones. The response parameter often adopted to evaluate cytostasis is TGI (concentration that totally inhibits the net cell growth but do not kill cells) – the test agent concentration able to exert total growth inhibition. Very high drug concentrations are unfortunately frequently used in the field of anticancer drug research. There are numerous examples of the use of cisplatin at concentrations of 10 - 50 μM (see [23] for a discussion) and 5FU at concentrations > 500 μM [24]. However, in these cases the drug exposure should be short since for instance 5FU will reach these concentration in plasma after a short bolus injection [25], but has a half-life of 10-15 min, while also in the FOLFOX and FOLFIRI protocols high concentrations are reached, even for a longer period. Similarly at standard doses of 50-100 mg/m^2 cisplatin, concentrations may peak between 10-20 μM [26, 27] but rapidly decline (half-life < 1 hr). The average IC_{50} values for both these drugs are 1-10 μM in the cell lines in the NCI60 panel at 48 h exposure, but for 5FU increase to 200-400 μM and for cisplatin up to 200 μM at a short 1-h exposure [28]. This means that *in vitro* experiments with these drugs at high concentration should be limited to a short exposure time. Microtubule interacting agents such as paclitaxel are also occasionally used at concentrations 100-fold more than their IC_{50} values [29]. The use of high drug concentrations for a prolonged period is convenient since massive apoptosis of cultured tumor cells is generally induced within 24 hours, a time frame that is ideal for conducting *in vitro* experiments. Induction of acute apoptosis by DNA damaging drugs using high drug concentrations is the subject of a large

number of studies. A PubMed search for cisplatin, apoptosis and mechanism generates 1618 hits (June 2017). The rate of publication of papers examining cisplatin-induced apoptosis has increased dramatically during recent years and these studies may represent a questionable use of valuable research resources. This problem has been pointed out by different investigators over the years [30-32], but the discussion has had limited impact on research directions. At even higher drug concentration, DNA damaging drugs have been reported to result in a phenomenon referred to as "programmed necrosis" [33]. The relevance of this death mode has been disputed [34].

The use of high drug concentrations will also accentuate alternative target effects that are unlikely to occur at drug concentrations that can be achieved *in vivo* at oral administration or a slow-release form. Cisplatin shows considerable reactivity with proteins due to its electrophilicity toward methionine, cysteine and histidine residues; protein adducts may therefore constitute the vast majority of cisplatin adducts in exposed cells [35, 36]. Protein adducts, usually irreversible, are expected to induce changes in cellular homeostasis only when accumulating over a certain threshold. The use of cisplatin concentrations that induce extensive formation of protein adducts is likely to lead to effects that are irrelevant to the therapeutically-relevant mechanism of action of this drug. Although such mechanisms may be of academic interest in terms of understanding apoptosis modes, they will not be relevant to understanding intrinsic and acquired cisplatin resistance.

The final-take home message coming from a critical analysis of the literature is that working with plasma drug concentration is more biologically reliable than working with high concentrations; and this appears to be true for different compounds.

(ii) The use of pharmacologically-relevant drug concentrations for novel compounds

Whilst the use of pharmacologically-relevant drug exposure parameters is easily defined for established drugs or drugs in advanced stages of preclinical/clinical evaluation, this is clearly

difficult or even impossible for novel compounds entering the drug discovery process for the first time. In this case, the design of the experiments should simply be determination of the IC_{50}/GI_{50} values and this is typically done using continuous drug exposures (48-72 h) in the first instance. The initial purpose is to reject compounds that do not have activity. Various selection criteria can be applied depending on the drug discovery strategy being pursued. For example, in the case of targeted anticancer drug development, drugs should be selected with cells expressing the target over those that do not. IC_{50} values should be comparable or lower than that of existing therapeutics (if they exist) or compounds with closely related mechanisms is a key decision point. However, it should be considered that mutation of the target may lead to resistance to the targeted therapy.

For compounds following the phenotypic drug discovery route, comparable activity to clinically approved reference anticancer drugs (that are chemically related for example) together with evidence of selectivity towards the tumor as opposed to non-cancer cell lines is appropriate [37, 38], although it has to be acknowledge that most cytotoxics do not discriminate between tumor and normal cells. In both cases, the purpose of the *in vitro* chemosensitivity assay is to generate sufficient evidence to select a small number of compounds to progress into the next phase of testing and in this case, the use of pharmacologically-relevant drug exposure parameters assumes less significance.

(iii) The identification of appropriate exposure times

It is important to define exposure time of compounds under study. Adequate exposure times need to consider both the known mechanism of action of the drug and pharmacokinetic parameters. The cytotoxic activity of certain drugs depends mainly on DNA replication, but it may not been necessary to treat cells for a period corresponding to one doubling time. This is the case for camptothecins and anthracyclines, DNA topoisomerase I and II inhibitors that rapidly stabilize the enzyme-DNA cleavable complex, leading to interference with normal DNA

functions. The antiproliferative activity of taxanes (e.g., paclitaxel) is due to stabilization of microtubuli, resulting in inhibition of the mitotic process. Prolonged treatment times are therefore required to achieve exposure in the critical G2/M phase of the cell cycle. For cisplatin, a short term exposure (1 or 2 h) is sufficient to generate DNA mono-adducts that are then stabilized or repaired over time. In experiments with targeted agents, a durable inhibition of the target is usually needed so that prolonged exposure times are mandatory.

This additional level of complexity to the problem of exposure time is presented by issues of drug uptake and metabolism. For instances, lipophilic drugs are usually rapidly taken up by cells and can accumulate intracellularly. For most drugs, especially investigational drugs, it is recommended that different exposure times are tested. Additionally, when making comparisons between tumor cell lines it is advisable to consider treating cells according to a number of doubling times, especially if isogenic pairs of cell lines are used to investigate the role of a potential gene or pathway in the mechanism of action of the drug.

(iv) The correct use of solvents to reconstitute pure compounds

All drugs, whether established or novel unknown compounds have to be dissolved in an appropriate solvent and appropriately diluted for use in *in vitro* chemosensitivity assays. Ideally, this should be an aqueous-based buffer but currently many novel compounds are insoluble in aqueous buffers. Therefore, dimethylsulphoxide (DMSO) or ethanol are widely used. It has been recently pointed out that this simple but fundamental issue in pharmacology has been neglected when performing preclinical studies, some of which are reported in high impact peer-reviewed journals. One can classify compounds as (i) soluble and solutions can be made in aqueous buffers; (ii) insoluble and the drug can be solubilised in either DMSO or ethanol; (iii) insoluble and it is recommended that the drug is prepared as a suspension. One should refrain from DMSO or ethanol, when the drug is water-soluble. A case in question concerns cisplatin that according to a recent paper by Hall *et al*. [39], has been incorrectly

dissolved in DMSO providing results that are misleading and clinically irrelevant. When dissolved in DMSO, cisplatin generates adducts that are different from those generated upon saline dissolution or when the drug is formulated for clinical use. Thirty years ago, Sundquist *et al*. reported about the species generated when cisplatin was dissolved in DMSO [40]. This observation was corroborated later in a publication in which adducts structurally distinct from those generated by correctly dissolved cisplatin are described [41]. In principle, cisplatin can be dissolved in water, but this generates species with altered cytotoxicity. Early evidence was presented about the possible use of "aquated" cisplatin in an attempt to increase the delivery of cisplatin to tumors [42]. However, such an approach resulted in increased nephrotoxicity in preclinical animal models. Thus, it appears reasonable that some discrepancies in the literature regarding platinum drugs might be dependent on the wrong choice of solvent, suggesting the need for minimal standard information of drug preparation for all papers in which drugs are used.

An important point in making solutions in pharmacological studies is protein binding, which can be reversible and irreversible. Sometimes protein binding is an advantage but more often a disadvantage. Also the type of protein is important. Protein binding can be promiscuous or specific. An excellent example which clearly affected drug development is the binding of UCN-01 to alpha-acid glycoprotein, which is not present in culture media, while it is abundant in human blood, where UCN-01 is almost completely inactivated. This means that in the early phase of drug development, protein binding (human and animal albumin, alpha-acid-glycoprotein, etc.) should be quantified. On the other hand proteins seem important in the intestinal uptake of several of the novel TKI as was demonstrated in the CaCo-2 model [43]. In the clinic this is seen with several drugs that show a better bioavailability when taken with food. For most TKI protein binding is > 95%, but seems reversible.

The use of pharmaceutical compounds *versus* pure compounds is also an important point that should be considered because clinical preparations may contain excipients that may affect cell response to the active compound.

For the clinically available antitumor agents, solvents and solubility have been optimized during the course of preclinical and clinical development studies. Drug development efforts have highlighted that the effect of the solvent on the activity of the drug under study is crucial, and it should be mandatory to provide details about the proper use of drugs in those manuscripts where *in vitro* or *in vivo* drug activity is tested; this includes a clear description of the source of serum. This would be helpful to improve the quality of the published literature as well to increase the translatability of the preclinical findings.

(v) Compound stability *in vitro*

A frequently underestimated aspect of pharmacology is drug stability throughout the duration of the *in vitro* assay [11, 44], especially those that use continuous drug exposures [12]. The stability of a number of cytotoxic drugs in cell culture media has been documented in the literature [44], but is frequently ignored with potentially relevant consequences. As an example, the selection of novel compounds for further evaluation is often based upon potency but if continuous drug exposures are used, the most active in a series of compounds may not necessarily be the most potent. In this hypothetical example, consider two compounds, one of which is stable *in vitro* over a prolonged period (compound A) and the other is highly unstable *in vitro* (compound B) with a half-life of less than one hour. Following continuous drug exposure, compound A had a lower IC_{50} value and this would naturally be selected in preference to compound B, but this conclusion is potentially misleading as compound B may actually be the most potent compound *in vitro* based upon C x T parameters for the active principle. Taking compound stability into consideration early on in the drug development process does however introduce logistic and technical challenges, but it is important to

acknowledge the potential limitations of interpreting structure activity relationships when continuous drug exposures are used. The impact of this problem can be reduced if other decision points such as selectivity for cancer *versus* non-cancer cells are taken into account as drug instability caused by chemical breakdown should be the same in both cell types. In addition, a simple approach to examine drug stability may be represented by wash out experiments. Another example constitutes the chemical instability of 5-aza-2'-deoxycytidine (Decitabine). To avoid decreased activity during repetitive freeze-thawing, this compound should be frozen in aliquots. Finally, it should also be stressed that breakdown of compounds *in vitro* does not always lead to inactive products (Figure 3). In the case of the alkylating agent ThioTEPA for example, it breaks down *in vitro* to the product TEPA which is just as active as the parent compound [45, 46].

This example leads us to comment on the *in vitro* use of pro-drugs. Irinotecan or carboplatin are often used in cellular studies and since they are pro-drugs, they are not really suitable for *in vitro* experiments. A good choice would be to employ SN38 instead of irinotecan as the former is the active metabolite of irinotecan generated upon the action of carboxylesterases, unless investigating mechanisms of resistance to irinotecan, to which reduced levels of carboxylesterase can be a contributory factor [47]. Furthermore, cisplatin should be used instead of carboplatin because the two compounds form identical adducts [48], but carboplatin has a lower rate of activation than cisplatin. With regards to aza-2'-deoxycytidine the intrinsic instability of the compound has been addressed by developing stable pro-drugs such as SGI-110.

Bio-activation is fundamentally essential for cyclophosphamide to exert its cytotoxic effects. Cytochrome P450s, (CYPs) mainly 2B6 and 3A4, oxidize cyclophosphamide to 4-hydroxy-cyclophosphamide (Figure 3) in the liver and this metabolite can subsequently enter cells and decompose to phosphoramide mustard, the ultimate active agent [49]. Therefore, for investigating the effects of cyclophosphamide *in vitro*, the 4-perhydroxy-derivatives must be

used, which spontaneously release 4-hydroxy-cyclophosphamide, which is not stable. Accordingly, for ifosfamide, 4-perhydroxyifosfamide must be used in cell culture experiments. Many publications on cyclophosphamide do not explicitly state if cells were incubated with the perhydroxy-derivative or cyclophosphamide itself [50].

(vi) Tumor microenvironmental factors

It is widely recognized that cell culture conditions do not mimic the complexity of tumor biology and it is questionable as to whether or not this is the real purpose of *in vitro* testing. The inherently flexible design of *in vitro* assays does allow the influence of tumor biology on pharmacology to be explored systematically in a controlled manner. This is particularly true for the tumor microenvironment. Together cellular heterogeneity, the physiological changes induced by a poor and inefficient blood supply and elevated interstitial fluid pressure can modify various aspects of anticancer drug pharmacology [11]. Understanding these factors is important in the design of *in vitro* assays that determine the impact of the tumor microenvironment on drug activity and cellular response. Three dimensional models may play a role in this context, but it is important to acknowledge that two dimensional models can also provide valuable information regarding the impact of specific microenvironmental factors on the pharmacology of anticancer drugs. Numerous examples exist in the literature where the effects of physiological factors such as hypoxia and acidosis on drugs have been evaluated [51, 52], but it is again important to stress that physiologically relevant parameters should be used. For example, the extracellular pH (pHe) in tumors is generally acidic (pHe typically ranges from 6.6 to 7.1) whereas an intracellular pH (pHi) of 7.4 is slightly higher than that in normal cells (pHi around 7.2) [53, 54]. This shift in pH gradients in cancer cells has profound biological and pharmacological implications [55-58] and it is therefore essential that physiologically relevant pH conditions are employed. Studies using more acidic pH values therefore need to be interpreted with caution.

Based upon the points raised in the above paragraphs, the following recommendations can be made. These are as follows:

- When data are available concerning the pharmacokinetic parameters achievable in humans or rodents, they should be used to guide the selection of concentrations to use in the *in vitro* setting.
- For novel compounds where pharmacokinetic data is not available, determination of IC_{50}, GI_{50}, TGI or LC_{50} following continuous drug exposure is an appropriate starting point.
- For compounds that are designed to target specific biochemical pathways, the use of cell lines where the target is well characterized or has been genetically manipulated is appropriate.
- For compounds where the mechanism of action is not known, comparison of the activity of the test compound against a chemically related standard agent (reference compound) and/or comparative activity against cancer as opposed to non-cancer cells is desirable. Often the NCI-60 cell line panel is a good starting point to find efficacy of drugs with similar structure. Data on all drugs tested by the NCI (a few 100,000) are available or will shortly become available. The COMPARE program often gives a suitable first insight in mechanism of action.
- Appropriate solvents should be employed to ensure complete solubility and the maintenance of the original chemical structure of the compound. Reporting of the preparation of compounds for use *in vitro* should be clear and precise in all publications.
- Characterization of physico-chemicals properties will help to define the appropriate solvent, but can also predict several important pharmacokinetic properties, such as drug penetration, volume of distribution [59].

- Compound stability should be taken into consideration when interpreting chemosensitivity data and the use of additional endpoint apart from potency to select compounds for further analysis should be considered. Freshly-prepared compound stocks and dilutions should be used in assays but where compound supply is limited; the preparation of stocks that are batched out and maintained at -20 °C or lower is desirable.
- Pro-drugs cannot be investigated in the same way as other drugs in experiments with the cell lines. Instead the active metabolite itself or compounds spontaneously releasing the active metabolite must be used.
- The endpoints used to measure cellular response can be tailored to the type or number of compounds under development; assay and assay conditions (length of drug exposure, day of drug addition, correction for absorbance/fluorescence/ cell count at day of drug addition) should be included. Just mentioning the commercial name of an assay is not sufficient.
- With regards to testing the effects of specific features of the tumor microenvironment (e.g., acidic pHe and reduced oxygenation conditions), it is important to use physiologically relevant conditions in two-dimensional culture systems.
- A number of consideration points to optimize the experimental design are listed in Table 1.

The experimental model: general issues

Over the years there has been an increased tendency to validate the experimental models to achieve specific objectives. The most recent example being the use of authenticated cell lines to eliminate the possibility of cross contamination of cultures by cells such as the HeLa cell line [60]. In contrast, evaluation of the appropriate use of antitumor compounds has been left behind and continues to rely on researcher choice and, ultimately, on the judgement of reviewers. Such phenomena need to be addressed, as we have long since moved from a time when

pharmacological preclinical research was conducted by a relatively small number of scientists carrying out preclinical drug development to a scientific context where many academic and commercial groups employ multiple pharmacological approaches designed to target biological alterations associated with tumor pathogenesis, progression and aggressiveness.

Thus, given the heterogeneous background of researchers employing drugs in their experiments, there are some important issues related to the experimental model that should be taken into account. When assaying cell sensitivity to certain drugs, it may be desirable to employ proliferating cells unless the experimental design implies that cells should not proliferate under the tested conditions. Cell proliferation is necessary for the cells to process the damage done to them by drugs, although this may depend on the type of damage. When DNA damage is not processed, the cells may turn out to be resistant to the drug (or become tolerant of drug-induced lesions) because they do not grow or undergo only a limited number of cell divisions during the time of the experiment. This is particularly important both for conventional anticancer agents inhibiting DNA-related functions or cell division and for agents targeting alterations associated with cell proliferation (e.g., EGF receptor). Low cellular proliferation rates and quiescence are however physiologically relevant conditions within hypoxic and poorly perfused tumor microenvironments. In the case of drug design it is desirable that new compounds target the hypoxic tumor microenvironment and activity against slowly proliferating cells [52, 61]. An additional caveat is represented by cells that do not proliferate rapidly, such as chronic lymphocytic leukaemia cells. Whilst these cells replicate very slowly, they are resistant to apoptosis as a consequence of MCL-1 expression. Agents which down-regulate MCL-1 expression (e.g., CDK9 inhibitors) impact cell survival and so measurement of apoptosis induction becomes the critical assay endpoint [62]. Similarly, normal tissues will contain a mix of proliferating undifferentiated cells and non-proliferating differentiated cells and therefore studies reporting the response of non-cancer cells need to be carefully interpreted. When interpreting observations where the IC_{50} of novel agents against tumor cells is compared

to normal cells, it is important to include standard agents in the study to serve as a "yardstick" against which the activity of new agents can be measured. If the new agent performs better than the established drug under identical experimental conditions, then there is a reasonable case for selecting this compound for further development.

An experimental model that has attracted major attention in recent years is represented by cancer stem cells (CSCs), a cell fraction endowed with self-renewal, differentiating and tumor initiating properties being responsible for tumor initiation, invasive growth, metastasis and drug resistance [63]. Although CSCs have been identified in several tumor types, the precise phenotypic and functional features of CSC have been well defined only in a limited number of studies, predominantly leukemia [64, 65], so that the use of preclinical models of CSCs, especially *in vitro* requires major attention (see below).

Testing cell sensitivity to drugs in *in vitro* assays: a variety of tests

As mentioned earlier, several end points are widely used to measure the effects of treatments on cell lines *in vitro* and these can be broadly divided into clonogenic and non-clonogenic assays. These assays do not only differ in technical nature, but also measure different cell fates. The clonogenic assay is a classical method to measure the response of cells following drug exposure [4]. The advantage of this assay is its ability to integrate different outcomes (apoptosis, necrosis, mitotic catastrophe, senescence) into colony forming ability as a measure of replicative potential. Although one is testing the ability of single cells/small cell numbers to survive brief exposure and retain proliferative capacity, the obtained data may resemble the scenario post tumor resection. The most commonly used type of endpoint assay however is the non-clonogenic assays largely because these can be semi-automated [5]. These assays (described in more detail in the next paragraph) are often referred to as determining the "cytotoxicity" of drugs which is not entirely correct; the results reflect the difference in cell number (or surrogate for cell number) between treated and control cultures due to effects on

cell growth/proliferation and/or cell death. Again, each endpoint has its own advantages and disadvantages and a detailed discussion of these can be found elsewhere [66].

With regards to non-clonogenic assays, a variety of assays exist whose suitability can be tailored to the specific objective of the study. Commonly used assays include the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [5], its derivatives (such as the XTT, WST), and Alamar Blue assay and the sulforhodamine B (SRB) assay [64] [6]. In the MTT/XTT assays, the tetrazolium salts are reduced in the mitochondria of viable cells to generate formazan products. Following solubilization of insoluble formazan products in organic solvents (MTT assay), the absorbance of the resulting solution can be determined and this is proportional to the number of metabolically viable cells within the culture. Similarly, in the Alamar Blue assay, resazurin is metabolically reduced to resorufin by viable cells [67]. As these assays provide a measure of metabolic activity, the use of these assays to evaluate compounds that target cell metabolism should be carefully interpreted and validated using alternative endpoints. Furthermore, differences in the pHe of control and treated cultures led to a significant underestimation of cell survival in cells treated with interferon using the MTT assay [68] and it is strongly recommended that the conditioned culture medium used to culture the cells is replaced with fresh medium immediately prior to the addition of MTT. This will apply to all assays that depend upon metabolic read outs to measure cellular response. In the SRB assay [6], it is possible to measure the dye binding to cellular proteins, again providing an indication of cell growth inhibition by treatment. MTT, Alamar blue and SRB assays provide surrogate indications/biomarkers of cell number. Many other assays are also available to assess cell sensitivity to drugs, e.g. the CellTiterGlo luminescent cell viability assay based on quantitation of the ATP present, a further indicator of metabolically active cells. It should be recognized that due to their metabolic properties quite a few drugs increase the intracellular concentration of ATP. An example is gemcitabine [69]. A simple alternative is staining with crystal violet, which is very useful in 96-wells plate assays, as well as to count stained colonies.

In the past, the activity of many compounds has been tested by microscopically counting viable cells using Trypan blue which is taken-up by dead cells, but excluded by intact membranes of viable cells. As mentioned above, the clonogenic assay [5] is adopted to determine the effects of short-term exposure on cell survival and colony formation. However, the clonogenic assay is time consuming and less suitable to large scale screenings, but remains very useful to detect cell death. Finally and crucially, an irrefutable measure of how a test agent may affect net cell numbers can be determined by simply cell counting after fixed exposure periods; cell counting allows direct determination of inhibition of cell growth, and when carried out with an automatic machine can provide very reproducible results.

Testing cell sensitivity to drugs in *in vitro* assays: 3D models

The vast majority of studies of the response to anticancer drugs are carried out using subconfluent monolayer cultures, conditions quite distinct from the situation in solid tumors in *vivo*. One strategy to attempt to mimic *in vivo* conditions is to use multicellular tumor spheroids (MCTS) [70, 71], aggregates of tumor cells formed *in vitro*. An alternative assay employed V-bottomed plates, in which cells form aggregates as well and resemble 3-dimensional spheroid. The advantage of such a system is that it can be evaluated using standard MTT and SRB assays [72]. The drug sensitivity properties resemble that of MCTS, as well as drug penetration. The sensitivities of MCTS and V-bottom cultures to anticancer drugs is generally lower than the sensitivities of monolayer cultures [73, 74]. This is true both for DNA damaging drugs and microtubule interacting agents [70, 75]. The major effect of cisplatin exposure of MCTS is senescence, whereas apoptosis is only observed in proliferating cell populations in peripheral cell layers [75]. The general insensitivity of MCTS to cisplatin and other anticancer drugs is likely a consequence of limited drug penetration and the presence of hypoxic, non-proliferating cell populations. The MCTS model has been technically improved, allowing spheroids of

homogeneous size to form in multiwell plates [70], and the model is in our opinion attractive for studies of the response of solid tumor cells to anticancer agents.

Three-dimensional models have been employed for *in vitro* assays using CSCs or putative CSCs. Indeed, the setting up of reliable drug sensitivity assays is critical because the true nature of CSCs is their tumor initiating ability that can be truly assayed only *in vivo* and due to the fact that the best condition would be to use tumor specimens as a source of cells maintaining stem-like features. However, isolation of CSCs from clinical specimens is not always successful [76]. In principle, once the stemness of a peculiar model has been established, drug sensitivity can be assessed by testing drug effects on spheres [77]. In fact, CSCs are capable of growing independently of anchorage (i.e. as spheres) in serum-free medium added with growth factors. In this context, there are several caveats to consider. For example, growth factors activate survival pathways that may influence response to drugs of different classes. Therefore, it remains difficult to standardize cell sensitivity assays of CSCs.

Alternatively, organoid cultures represent an appealing experimental model for testing antitumor agents because of the potential to model cancer *in vitro,* somehow respecting the complexity of the disease and recapitulating the three-dimensional tumor organization [78]. The use of this appealing technology is still at its infancy, although it appears that it can be developed for drug testing by the concomitant employment of different tests to assess cell viability or growth.

3D models: Drug penetration

A major limiting factor in the effectiveness of chemotherapy is poor and inadequate extravascular penetration of anticancer drugs [11]. Whilst drug penetration barriers have been identified using drugs that are naturally fluorescent or radioactively labelled [11], the development of the multicell layer cell culture models have enabled the kinetics of drug penetration to be determined using routine analytical techniques [79]. Whilst good penetration

of drugs into avascular regions of tumors is desirable in all cases, it is an absolute requirement for drugs that are designed to target the hypoxic fraction of tumors [52, 61, 80]. The kinetics of drug penetration has been combined with mathematical modelling to generate *in silico* models that can help drug development pathways in terms of selecting and designing compounds that can penetrate into such regions of tumors [81, 82].

Testing of drug combinations

A common goal of preclinical researchers is to discover synergistic interactions between drugs. The postulated rationale for the use of combination treatment regimens includes: reduction of single agent doses in order to minimize adverse systemic toxicity and spare normal tissues [83, 84]; target more tumor cells, bearing in mind the heterogeneous nature of malignant disease; avoid or delay emergence of acquired drug resistance. However, in combination therapies it is often neglected that a reduction of the dose of a drug invariably results in a decreased efficacy, while the efficacy of a combination is often not compared to the efficacy of a single drug at its maximal tolerated dose (MTD). This means that a combination does not make sense when its efficacy is similar or less than that of each single drug at its MTD. Moreover, in a combination the drugs may not only enhance the antitumor effect, but also the toxicity. Therefore toxicity of the combination should also be compared to that of the single drug at its MTD.

Nevertheless there are many combinations, which fulfil these criteria and have successfully been translated from the cell culture to the mouse model to the patients and clearly improved the efficacy compared to the single drugs [85]. Many approaches have been described over the years aimed at identification of synergistic interactions, including the simple Bliss independence method re-proposed by Kern in 1989 [86], the Loewe isobologram, Webb fractional product concept of synergy and the median drug effect methodology developed by Chou and Talalay [83, 87]. In this method, a combination index (CI) is calculated at various fraction affected (FA) in which a FA of 0 is no effect and a FA of 1 is complete growth

inhibition. The Chou and Talalay method is widely used for determination of synergy, additivity or antagonism between drugs in combination. However, the method is often used incorrectly; e.g. it does not make sense to demonstrate synergism at a total of 25% growth inhibition level (FA = 0.25), since that means that the tumor still grows at a 75% rate compared to untreated; even an increase to 50% inhibition (FA = 0.5) still means 50% growth of a tumor. The normal application of the method is limited to 95% growth inhibition (FA = 0.95) and cell kill cannot be included in the formula, since it does not allow negative values nor values of FA>1.0. It is recommended that in the application of this method only FA values between 0.5 and 0.95 are included [84], while an adaptation of the methods also allows evaluation of cell kill.

Unfortunately, there are still papers published where the improved effect of the combination *in vitro* has been assessed in the absence of adequate mathematical analysis, or an incorrect use of mathematics. Although discussion of the best method to evaluate drug interaction is out of the scope of this manuscript, we would like to highlight the importance of a correct application of one of the available methods for assessing synergism in *in vitro* tests for drug interactions. With few exceptions (outlined above), such studies should not rely merely on statistical analyses by (for example) the Student's t (or similar) tests.

Concluding remarks

The current scenario of pharmacological science implies both pharma-driven efforts and academic contribution to innovate drug development and to optimize therapeutic approaches toward the path of personalized medicine. The translatability of preclinical research on antitumor agents is only in part successful for several reasons, including issues related to changes in strategies in academic anticancer drug discovery [3] and experimental models and experimental design applied in the preclinical setting [88]. A pertinent example is provided by EGFR inhibitors finally discovered to act on the mutant receptor [89]. An improvement in

terms of preclinical research translatability may be provided also by the application of more stringent criteria and transparency throughout preclinical phases of testing.

A recent publication authored by Alan Eastman has highlighted the importance of the appropriate use of cytotoxicity assays and combination models to improve anticancer drug development [90]. The author highlights the high failure rate of anticancer agents considering possible drug or experimental model-related reasons. The latter issue is still forefront; also taking into account that murine models only in part mirror the complexity of human tumors.

Additionally, an important point is the development of compounds with a high therapeutic index, capable of killing cancer cells while sparing normal ones. Recently, non-cancer cells from different tissues have become available and their use in *in vitro* testing is helpful, especially when cells are derived from tissues normally involved in dose-limiting toxicities.

There is an urgent need to improve the quality of preclinical results obtained with new compounds and with clinically available agents. Researchers in the field of pharmacology generally know by virtue of their training about the relevance of solvents, concentrations, use of drugs versus pro-drugs, inclusion of pertinent controls and stringent, reproducible assay conditions in cellular pharmacology studies. However, researchers in the field of molecular biology often lack this background. In this context, attention should be paid (in addition to cell culture conditions) to cell behavior and to reagents used for cell culture [91]. In fact, the most widely used cell culture supplement (fetal bovine serum) is very complex, its bioactive compounds vary between batches and may affect the outcome of cell sensitivity tests. However, it should not be difficult for beginners to consider all the possible sources of variability and to set up antitumor pharmacology experiments properly, given that all the scientific community can access a lot of information about compound solubility, stability and sensitivity assays through multiple web-available sources. We hope that the issues raised herein offer the opportunity to reflect upon relevant points and act as “pocket” guidelines to motivate good

practise in design of pharmacology experiments, and to include in their articles the necessary information for the tested agents.

Compliance with Ethical Standards:

Conflict of Interest: the corresponding author declares – on behalf of all authors – that the authors have no conflict of interest.

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References

1. Tajeddine N, Galluzzi L, Kepp O, Hangen E, Morselli E, Senovilla L, Araujo N, Pinna G, Larochette N, Zamzami N, Modjtahedi N, Harel-Bellan A, Kroemer G (2008) Hierarchical involvement of Bak, VDAC1 and Bax in cisplatin-induced cell death. Oncogene 27:4221-4232

2. Mantovani F, Piazza S, Gostissa M, Strano S, Zacchi P, Mantovani R, Blandino G, Del Sal G (2004) Pin1 links the activities of c-Abl and p300 in regulating p73 function. Mol Cell 14:625-636

3. Hendriks HR, Govaerts AS, Fichtner I, Burtles S, Westwell AD, Peters GJ (2017) Pharmacologically directed strategies in academic anticancer drug discovery based on the European NCI compounds initiative. Br J Cancer 117:195-202

4. Righetti SC, Perego P, Carenini N, Zunino F (2008) Cooperation between p53 and p73 in cisplatin-induced apoptosis in ovarian carcinoma cells. Cancer Lett 263:140-144

5. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1:2315-2319

6. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48:589-601

7. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107-1112

8. Kahn J, Tofilon PJ, Camphausen K (2012) Preclinical models in radiation oncology. Radiat Oncol 7:223-717X-7-223

9. Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57:727-741

10. Rossi C, Gasparini G, Canobbio L, Galligioni E, Volpe R, Candiani E, Toffoli G, D'Incalci M (1987) Doxorubicin distribution in human breast cancer. Cancer Treat Rep 71:1221-1226

11. Minchinton AI, Tannock IF (2006) Drug penetration in solid tumours. Nat Rev Cancer 6:583-592

12. Hockel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93:266-276

13. Lanvers C, Hempel G, Blaschke G, Boos J (1998) Chemically induced isomerization and differential uptake modulate retinoic acid disposition in HL-60 cells. FASEB J 12:1627-1633

14. Serova M, Tijeras-Raballand A, Dos Santos C, Martinet M, Neuzillet C, Lopez A, Mitchell DC, Bryan BA, Gapihan G, Janin A, Bousquet G, Riveiro ME, Bieche I, Faivre S, Raymond E, de Gramont A (2016) Everolimus affects vasculogenic mimicry in renal carcinoma resistant to sunitinib. Oncotarget 7:38467-38486

15. Gotink KJ, Broxterman HJ, Labots M, de Haas RR, Dekker H, Honeywell RJ, Rudek MA, Beerepoot LV, Musters RJ, Jansen G, Griffioen AW, Assaraf YG, Pili R, Peters GJ, Verheul HM (2011) Lysosomal sequestration of sunitinib: a novel mechanism of drug resistance. Clin Cancer Res 17:7337-7346

16. Gotink KJ, Broxterman HJ, Honeywell RJ, Dekker H, de Haas RR, Miles KM, Adelaiye R, Griffioen AW, Peters GJ, Pili R, Verheul HM (2014) Acquired tumor cell resistance to

sunitinib causes resistance in a HT-29 human colon cancer xenograft mouse model without affecting sunitinib biodistribution or the tumor microvasculature. Oncoscience 1:844-853

17. Peters GJ, Lankelma J, Kok RM, Noordhuis P, van Groeningen CJ, van der Wilt CL, Meyer S, Pinedo HM (1993) Prolonged retention of high concentrations of 5-fluorouracil in human and murine tumors as compared with plasma. Cancer Chemother Pharmacol 31:269-276

18. Da Silva CG, Honeywell RJ, Dekker H, Peters GJ (2015) Physicochemical properties of novel protein kinase inhibitors in relation to their substrate specificity for drug transporters. Expert Opin Drug Metab Toxicol 11:703-717

19. van der Veldt AA, Smit EF, Lammertsma AA (2013) Positron Emission Tomography as a Method for Measuring Drug Delivery to Tumors in vivo: The Example of [(11)C]docetaxel. Front Oncol 3:208

20. Bahce I, Smit EF, Lubberink M, van der Veldt AA, Yaqub M, Windhorst AD, Schuit RC, Thunnissen E, Heideman DA, Postmus PE, Lammertsma AA, Hendrikse NH (2013) Development of [(11)C]erlotinib positron emission tomography for in vivo evaluation of EGF receptor mutational status. Clin Cancer Res 19:183-193

21. Qazzaz ME, Raja VJ, Lim KH, Kam TS, Lee JB, Gershkovich P, Bradshaw TD (2016) In vitro anticancer properties and biological evaluation of novel natural alkaloid jerantinine B. Cancer Lett 370:185-197

22. Stone EL, Citossi F, Singh R, Kaur B, Gaskell M, Farmer PB, Monks A, Hose C, Stevens MF, Leong CO, Stocks M, Kellam B, Marlow M, Bradshaw TD (2015) Antitumour benzothiazoles. Part 32: DNA adducts and double strand breaks correlate with activity; synthesis of 5F203 hydrogels for local delivery. Bioorg Med Chem 23:6891-6899

23. Berndtsson M, Hagg M, Panaretakis T, Havelka AM, Shoshan MC, Linder S (2007) Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. Int J Cancer 120:175-180

24. Akpinar B, Bracht EV, Reijnders D, Safarikova B, Jelinkova I, Grandien A, Vaculova AH, Zhivotovsky B, Olsson M (2015) 5-Fluorouracil-induced RNA stress engages a TRAIL-DISC-dependent apoptosis axis facilitated by p53. Oncotarget 6:43679-43697

25. van Groeningen CJ, Pinedo HM, Heddes J, Kok RM, de Jong AP, Wattel E, Peters GJ, Lankelma J (1988) Pharmacokinetics of 5-fluorouracil assessed with a sensitive mass spectrometric method in patients on a dose escalation schedule. Cancer Res 48:6956-6961

26. Himmelstein KJ, Patton TF, Belt RJ, Taylor S, Repta AJ, Sternson LA (1981) Clinical kinetics on intact cisplatin and some related species. Clin Pharmacol Ther 29:658-664

27. van Moorsel CJ, Kroep JR, Pinedo HM, Veerman G, Voorn DA, Postmus PE, Vermorken JB, van Groeningen CJ, van der Vijgh WJ, Peters GJ (1999) Pharmacokinetic schedule finding study of the combination of gemcitabine and cisplatin in patients with solid tumors. Ann Oncol 10:441-448

28. Vanarkotte J, Peters G, Pizao P, Keepers Y, Giaccone G (1994) In-vitro schedule-dependency of eo9 and miltefosine in comparison to standard drugs in colon-cancer cells. Int J Oncol 4:709-715

29. Lee LF, Li G, Templeton DJ, Ting JP (1998) Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). J Biol Chem 273:28253-28260

30. Borst P, Borst J, Smets LA (2001) Does resistance to apoptosis affect clinical response to antitumor drugs?. Drug Resist Updat 4:129-131

31. Brown JM, Attardi LD (2005) The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 5:231-237

32. Havelka AM, Berndtsson M, Olofsson MH, Shoshan MC, Linder S (2007) Mechanisms of action of DNA-damaging anticancer drugs in treatment of carcinomas: is acute apoptosis an "off-target" effect?. Mini Rev Med Chem 7:1035-1039

33. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18:1272-1282

34. Borst P, Rottenberg S (2004) Cancer cell death by programmed necrosis?. Drug Resist Updat 7:321-324

35. Gonzalez VM, Fuertes MA, Alonso C, Perez JM (2001) Is cisplatin-induced cell death always produced by apoptosis?. Mol Pharmacol 59:657-663

36. Ivanov AI, Christodoulou J, Parkinson JA, Barnham KJ, Tucker A, Woodrow J, Sadler PJ (1998) Cisplatin binding sites on human albumin. J Biol Chem 273:14721-14730

37. Faulkner AD, Kaner RA, Abdallah QM, Clarkson G, Fox DJ, Gurnani P, Howson SE, Phillips RM, Roper DI, Simpson DH, Scott P (2014) Asymmetric triplex metallohelices with high and selective activity against cancer cells. Nat Chem 6:797-803

38. Kaner RA, Allison SJ, Faulkner AD, Phillips RM, Roper DI, Shepherd SL, Simpson DH, Waterfield NR, Scott P (2016) Anticancer metallohelices: nanomolar potency and high selectivity. Chem Sci 7:951-958

39. Hall MD, Telma KA, Chang KE, Lee TD, Madigan JP, Lloyd JR, Goldlust IS, Hoeschele JD, Gottesman MM (2014) Say no to DMSO: dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res 74:3913-3922

40. Sundquist WI, Lippard SJ, Stollar BD (1987) Monoclonal antibodies to DNA modified with cis- or trans-diamminedichloroplatinum(II). Proc Natl Acad Sci U S A 84:8225-8229

41. Fischer SJ, Benson LM, Fauq A, Naylor S, Windebank AJ (2008) Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. Neurotoxicology 29:444-452

42. Zheng H, Fink D, Howell SB (1997) Pharmacological basis for a novel therapeutic strategy based on the use of aquated cisplatin. Clin Cancer Res 3:1157-1165

43 Honeywell RJ, Fatmawati C, Buddha M, Hitzerd S, Kathman I, Peters GJ (2015). Adaptation of a human gut epithelial model in relation to the assessment of clinical pharmacokinetic parameters for selected tyrosine kinase inhibitors. ADMET & DMPK 3: 51-67

44. Wagner A, Hempel G, Gumbinger HG, Jurgens H, Boos J (1999) Pharmacokinetics of anticancer drugs in vitro. Adv Exp Med Biol 457:397-407

45. Jacobson PA, Green K, Birnbaum A, Remmel RP (2002) Cytochrome P450 isozymes 3A4 and 2B6 are involved in the in vitro human metabolism of thiotepa to TEPA. Cancer Chemother Pharmacol 49:461-467

46. Phillips RM, Bibby MC, Double JA (1988) Experimental correlations of in vitro drug sensitivity with in vivo responses to ThioTEPA in a panel of murine colon tumours. Cancer Chemother Pharmacol 21:168-172

47. Xu Y, Villalona-Calero MA (2002) Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Ann Oncol 13:1841-1851

48. Perego P, Robert J (2016) Oxaliplatin in the era of personalized medicine: from mechanistic studies to clinical efficacy. Cancer Chemother Pharmacol 77:5-18

49. Emadi A, Jones RJ, Brodsky RA (2009) Cyclophosphamide and cancer: golden anniversary. Nat Rev Clin Oncol 6:638-647

50. Ahlmann M, Hempel G (2016) The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy. Cancer Chemother Pharmacol 78:661-671

51. Kolosenko I, Avnet S, Baldini N, Viklund J, De Milito A (2017) Therapeutic implications of tumor interstitial acidification. Semin Cancer Biol 43:119-133

52. Phillips RM (2016) Targeting the hypoxic fraction of tumours using hypoxia-activated prodrugs. Cancer Chemother Pharmacol 77:441-457

53. Gallagher FA, Kettunen MI, Day SE, Hu DE, Ardenkjaer-Larsen JH, Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM (2008) Magnetic resonance imaging of pH in vivo using hyperpolarized 13C-labelled bicarbonate. Nature 453:940-943

54. Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV, Gillies RJ (2011) Imaging pH and metastasis. NMR Biomed 24:582-591

55. Oudin MJ, Weaver VM (2016) Physical and Chemical Gradients in the Tumor Microenvironment Regulate Tumor Cell Invasion, Migration, and Metastasis. Cold Spring Harb Symp Quant Biol 81:189-205

56. Kato Y, Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T, Baba Y (2013) Acidic extracellular microenvironment and cancer. Cancer Cell Int 13:89-2867-13-89

57. Swietach P, Vaughan-Jones RD, Harris AL, Hulikova A (2014) The chemistry, physiology and pathology of pH in cancer. Philos Trans R Soc Lond B Biol Sci 369:20130099

58. Larsen AK, Escargueil AE, Skladanowski A (2000) Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol Ther 85:217-229

59 Valko KL, Teague SP, Pidgeon C (2017). In vitro membrane binding and protein binding (IAM MB/PB technology) to estimate in vivo distribution: applications in early drug discovery. ADMET & DMPK 5:14-38

60. Almeida JL, Cole KD, Plant AL (2016) Standards for Cell Line Authentication and Beyond. PLoS Biol 14:e1002476

61. Hunter FW, Wouters BG, Wilson WR (2016) Hypoxia-activated prodrugs: paths forward in the era of personalised medicine. Br J Cancer 114:1071-1077

62. Walsby E, Pratt G, Shao H, Abbas AY, Fischer PM, Bradshaw TD, Brennan P, Fegan C, Wang S, Pepper C (2014) A novel Cdk9 inhibitor preferentially targets tumor cells and synergizes with fludarabine. Oncotarget 5:375-385

63. Clevers H (2005) Stem cells, asymmetric division and cancer. Nat Genet 37:1027-1028

64. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267-284

65. Dalerba P, Clarke MF (2007) Cancer stem cells and tumor metastasis: first steps into uncharted territory. Cell Stem Cell 1:241-242

66. Freshney RI (2016) Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 7th Edition, Wiley-Blackwell

67. Collins L, Franzblau SG (1997) Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob Agents Chemother 41:1004-1009

68. Jabbar SA, Twentyman PR, Watson JV (1989) The MTT assay underestimates the growth inhibitory effects of interferons. Br J Cancer 60:523-528

69. van Moorsel CJ, Bergman AM, Veerman G, Voorn DA, Ruiz van Haperen VW, Kroep JR, Pinedo HM, Peters GJ (2000) Differential effects of gemcitabine on ribonucleotide pools of twenty-one solid tumour and leukaemia cell lines. Biochim Biophys Acta 1474:5-12

70. Fayad W, Brnjic S, Berglind D, Blixt S, Shoshan MC, Berndtsson M, Olofsson MH, Linder S (2009) Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model. Int J Cancer 125:2450-2455

71. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK (2003) Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol Bioeng 83:173-180

72. Padron JM, van der Wilt CL, Smid K, Smitskamp-Wilms E, Backus HH, Pizao PE, Giaccone G, Peters GJ (2000) The multilayered postconfluent cell culture as a model for drug screening. Crit Rev Oncol Hematol 36:141-157

73. Sutherland RM, Eddy HA, Bareham B, Reich K, Vanantwerp D (1979) Resistance to adriamycin in multicellular spheroids. Int J Radiat Oncol Biol Phys 5:1225-1230

74. Olive PL, Durand RE (1994) Drug and radiation resistance in spheroids: cell contact and kinetics. Cancer Metastasis Rev 13:121-138

75. Frankel A, Buckman R, Kerbel RS (1997) Abrogation of taxol-induced G2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. Cancer Res 57:2388-2393

76. D'Arcangelo M, Todaro M, Salvini J, Benfante A, Colorito ML, D'Incecco A, Landi L, Apuzzo T, Rossi E, Sani S, Stassi G, Cappuzzo F (2015) Cancer Stem Cells Sensitivity Assay (STELLA) in Patients with Advanced Lung and Colorectal Cancer: A Feasibility Study. PLoS One 10:e0125037

77. Beretta GL, De Cesare M, Albano L, Magnifico A, Carenini N, Corna E, Perego P, Gatti L (2016) Targeting peptidyl-prolyl isomerase pin1 to inhibit tumor cell aggressiveness. Tumori 102:144-149

78. Drost J, Clevers H (2017) Translational applications of adult stem cell-derived organoids. Development 144:968-975

79. Phillips RM, Loadman PM, Cronin BP (1998) Evaluation of a novel in vitro assay for assessing drug penetration into avascular regions of tumours. Br J Cancer 77:2112-2119

80. Hicks KO, Pruijn FB, Sturman JR, Denny WA, Wilson WR (2003) Multicellular resistance to tirapazamine is due to restricted extravascular transport: a pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures. Cancer Res 63:5970-5977

81. Foehrenbacher A, Secomb TW, Wilson WR, Hicks KO (2013) Design of optimized hypoxia-activated prodrugs using pharmacokinetic/pharmacodynamic modeling. Front Oncol 3:314

82. Groh CM, Hubbard ME, Jones PF, Loadman PM, Periasamy N, Sleeman BD, Smye SW, Twelves CJ, Phillips RM (2014) Mathematical and computational models of drug transport in tumours. J R Soc Interface 11:20131173

83. Chou TC (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70:440-446

84. Bijnsdorp IV, Giovannetti E, Peters GJ (2011) Analysis of drug interactions. Methods Mol Biol 731:421-434

85. Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP (2000) Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther 87:227-253

86. Kern DH, Morgan CR, Hildebrand-Zanki SU (1988) In vitro pharmacodynamics of 1-beta-D-arabinofuranosylcytosine: synergy of antitumor activity with cis-diamminedichloroplatinum(II). Cancer Res 48:117-121

87. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27-55

88. Corsello SM, Bittker JA, Liu Z, Gould J, McCarren P, Hirschman JE, Johnston SE, Vrcic A, Wong B, Khan M, Asiedu J, Narayan R, Mader CC, Subramanian A, Golub TR (2017) The Drug Repurposing Hub: a next-generation drug library and information resource. Nat Med 23:405-408

89. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497-1500

90. Eastman A (2017) Improving anticancer drug development begins with cell culture: misinformation perpetrated by the misuse of cytotoxicity assays. Oncotarget 8:8854-8866

91. Baker M (2016) Reproducibility: Respect your cells! Nature 537:433-435

Table 1. Suggestions in the design of preclinical *in vitro* testing experiments

Choice of the compound concentration
After testing a wide range of concentrations, am I carrying out the relevant experiments using clinically meaningful concentrations?
Choice of the exposure and recovery time
Do I allow the cells to proliferate long enough to assess the treatment outcome?
Did I consider cell doubling time?
Did I match the drug exposure time to the retention of the drug *in vivo*, a high concentration only for a short time?
Choice of solvent
Is the compound water or fat soluble (lipid and non polar solvents)?
Is the solvent (e.g. DMSO or ethanol) used at concentrations that are non toxic for the cells?
Is the dissolved compound stable so that it can be stocked at low temperature (-20°C; -80°C)?
Does the compound show protein binding; is that reversible?
Choice of the compound
Is the compound stable?
Is the compound a pro-drug?
If I am using a pro-drug in vitro, can I substitute it with the active metabolite?
Choice of cell sensitivity assay
Does the endpoint of the assay addresses the experimental question correctly?
Does the method recommend and allow to correct for the number of cells at the time of drug addition?
Choice of experimental model
Does the experimental model express the target of interest?

Legends to figures

Figure 1. Basic experimental design for the conduct of *in vitro* chemosensitivity assays using adherent cell lines. The central bar is a time line and the key steps are placed on this time line. The procedures for conducting continuous and timed drug exposures are presented in panels A and B respectively. The procedures for suspension cultures are the same with the exception of the conditioning period in culture plates which isn't required. The endpoint here refers to non-clonogenic assays as clonogenic assays usually require longer times for colony formation to occur. Readings should be performed at the time of drug addition and at the endpoint. Intermediate time-points can be very informative since they give insight in the dynamics of drug response. With several of the novel assays, that do not require addition of a dye, it is possible to do these measurements.

Figure 2. Criteria for selecting drug concentrations and solvent choice for in vitro cell sensitivity assays. The two main conditions, i.e. use of established drugs or novel compounds are represented, besides a summary of the possible solvents.

Figure 3. Structure of selected drugs, prodrugs and their active metabolites. The alkylating agent ThioTEPA breaks down *in vitro* to TEPA which is equally active as the original compound. Cisplatin and carboplatin form identical DNA adducts. SN-38 is the active metabolite formed from irinotecan by carboxylesterases. Cyclophosphamide is converted to 4-hydroxycyclophosphamide by P450.