

Review

The role of pharmacology in anticancer drug development

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Abstract

Drug development consists of many sequential and parallel steps; failure in one of the steps can lead to discontinuation of the process. The process is time-consuming and very expensive, especially the clinical phase. In order to enhance cancer drug development in the 1980s, the National Cancer Institute (NCI) adopted a new screening system using 60 different tumour cell lines from various histologies. All standard drugs were tested in this panel and it is still open for testing of new chemical entities (NCE) of potential interest. The European NCI compounds initiative, a collaborative programme of the NCI, the Cancer Research Campaign (CRC; now CRUK) and the Pharmacology and Molecular Mechanism Group (PAMM) of the EORTC (European Organization on Research and Treatment of Cancer), was initiated in 1993. The programme aimed to help the NCI reducing its backlog of in vivo testing by further evaluation of interesting European compounds using a pharmacologically directed approach. Considerable multidisciplinary expertise in drug development was combined by the CRC and EORTC-PAMM: chemists, pharmacists, biologists, pharmacologists, oncologists. Selection criteria for European NCI compounds included novelty of the NCE, in vitro activity, if available in vivo and hollow fibre activity, and COMPARE negativity. Over a period of more than 20 years 95 out of approximately 2,000 reviewed compounds were selected. These compounds were put through a series of stepwise pharmacological tests comprising solubility (suitable formulation to administer the NCE to mice), feasibility to develop a simple analytical assay (usually HPLC), limited toxicology and angiogenic properties. This paper provides examples to illustrate the rigorousness of the elimination process of the compounds and discusses the way to improve the process by inclusion of more physico-chemical parameters.

Keywords

EORTC-PAMM; NCI; CRUK; *in vitro* screen; pharmacological approach; anticancer drug development; European NCI compounds

Introduction

Anticancer drug development is a collaborative effort. When a compound has been synthesised chemically or isolated from a specific source, e.g. a natural product, it will be characterised chemically, physically, pharmacologically, pharmaceutically, toxicologically and biologically, requiring the input from various disciplines. When a compound passes all these steps successfully it continues to clinical development [1]. A tightly controlled process precedes the first administration to a patient. This process is

subject to many regulations and requires approval by local hospital medical ethical review boards and national competent authorities. This first step is the Phase I clinical study, in which the safety, side effects and best dose of a drug are determined. The latter can be the maximum tolerated dose (MTD) for cytotoxics or the maximum effective dose (usually lower than the MTD) for compounds affecting signaling pathways. This dose is used to determine the antitumor activity of the agent in a Phase II study [1-3]. In the classical drug development setting, an active compound (antitumor activity comparable to that of standard therapy), will proceed to a randomised Phase III study in which the new drug will be compared to the standard therapy or placebo. However, with several novel compounds in which a target is defined, a Phase I study may be expanded to include only patients with predefined biomarkers [3]. Drugs can obtain market approval from Medicines agencies such as the EMA and FDA, based on an extensive risk/benefit ratio evaluation.

An increasing understanding of cancer biology leads to a heterogeneity at multiple levels (genetic, functional, tumour components, signalling pathways), which translates into an increasing number of products in the pipeline of pharmaceutical companies. This high number of compounds resulted in more Phase I, II and III clinical trials requiring even more patients, which led to extensively high costs for pharmaceutical industry and subsequently health care in society [4,5]. These numbers (costs and number of patients) are increasing in the last decades [6], while the rate of success in oncology drug development is limited, compared to other diseases [7]. In order to keep the costs as low as possible it is important that every aspect of the development process is critically reviewed, including optimisation of drug selection at an early stage.

The European Organization for Research and Treatment of Cancer (EORTC) is the largest European organization coordinating clinical studies in the development of new cancer therapies [8]. The National Cancer Institute (NCI) in Bethesda, MD, USA, developed in the 2nd half of the 1980s a 60-cell line panel (called NCI-60 panel throughout this paper), consisting of 60 cell lines representative for the 9 major tumour types, with the aim to select compounds that show selective potency against one of these malignancies [9]. This NCI-60 panel was offered to the scientific community to test the potency of Novel Chemical Entities (NCEs) and since that period hundreds of thousands of compounds have been submitted to the NCI screen, but not all passed the initial NCI selection procedure and were not tested. Several tested compounds showed an interesting profile and were candidates for further development e.g. *in vivo* testing, but early in this process it was recognized that too many compounds fulfilled the criteria for *in vivo* testing. In order to speed up the backlog in this development and since many compounds came from Europe, an international academic collaboration between both sides of the Atlantic was initiated in 1993, between the EORTC Research Branch, the Cancer Research Campaign (CRC, now CRUK) and the NCI, the so-called European NCI compounds initiative, which focused on compounds synthesised in Europe and showing interesting data in the NCI screen. CRC and EORTC set up a review committee to select European compounds for further development [10,11]. About 2,000 compounds were evaluated by the CRC-EORTC review committee in >20 years and 95 were selected for further evaluation. Further details on this process and the achievements have recently been summarised [10]. The purpose of this paper is to evaluate mechanistic details e.g. ADME (Absorption, Distribution, Metabolism, Excretion) in this process by describing the development of several compounds that either failed or were successful. Successful means moving into Phase I or eventually Phase II clinical studies. Failure may have been caused by obvious parameters, such as lack of potency, poor solubility, stability and other physico-chemical properties.

The process of European NCI compound evaluation

In the early 1990s, the screening's capacity of the NCI-60 panel was 10,000 compounds annually. NCEs, submitted by scientists from all over the world, are selected for screening based on structural diversity and novelty of the compound. In the initial program selected NCEs were tested at 5 different concentrations (varying over 5 logs: 10^{-4} – 10^{-8} or 10^{-5} – 10^{-9} M) and cells were exposed for 48 hours to the drug. Three parameters were determined for each drug: the GI50 (or IC50: 50 % growth inhibition), the TGI (total growth inhibition) and the LC50 (50 % cell kill or 50 % lethal concentration) (Figure 1) [12]. The results of the screen are reported as growth inhibition curves as shown in Figure 2 for compound NSC-625492. Many current drug sensitivity tests often denote these curves as viability curves. This is not a correct terminology, because growth inhibition is measured. The NCI data are summarized as mean graphs for GI50, TGI or LC50 values. These mean graphs depict whether a cell line is more or less sensitive than the GI50/TGI/LC50 mean of the test drug in the NCI-60 panel. Figure 3 shows the mean graphs of compound NSC-710305 [13], also known as Phortress, and a prodrug of Phortress 5F-203. These mean graphs demonstrate a tumour specific sensitivity for lung, ovarian, renal and breast cancer (bars to the right). Subsequent mechanistic studies were performed according to the guidelines published recently [14].

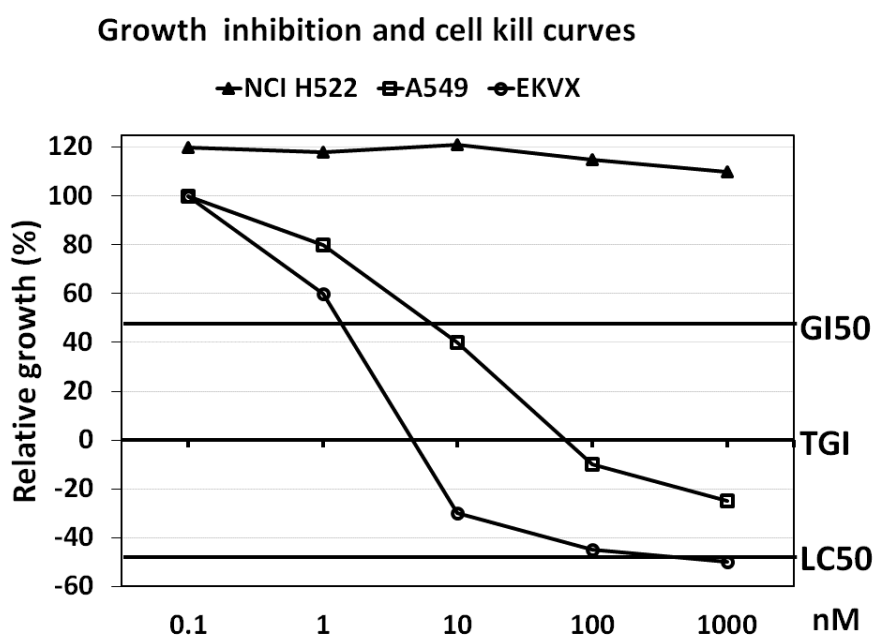


Figure 1. Example growth inhibition curves after 48 hours exposure to a hypothetical drug tested at 0.1 to 1000 nM in three lung cancer cell lines. Three parameters are shown: the GI50 (the concentration that gives 50 % growth inhibition, often also referred to as IC50), the TGI (the concentration that gives a total growth inhibition meaning that the number of cells at the end of the exposure is similar to that at the beginning) and the LC50 (the concentration that gives 50 % cell kill, meaning that at the end the number of cells is 50 % of the number of cells at the beginning). Values are calculated according to the NCI protocol:

https://dtp.cancer.gov/discovery_development/nci-60/methodology.htm

When positive, such as for Phortress, compounds were further screened in a human tumour xenograft (HTX) panel. However, the NCI screen yielded too many potential compounds for HTX testing, so that a fast low-cost preliminary *in vivo* screen was developed, the so-called hollow fibre assay (HFA) [15,16]. This assay combines advantages of *in vitro* screens (relatively fast, several tumour types together) and bypasses the disadvantages such as the poor predictive capacity for *in vivo* activity and lack of information on potential pharmacological properties. The latter are important to determine the amount of drug to be delivered [17]. The HFA appeared to predict for HTX activity [18,19].

Sensitivity to NSC-625492 in the NCI-60 cell line panel (source NCI DTP)

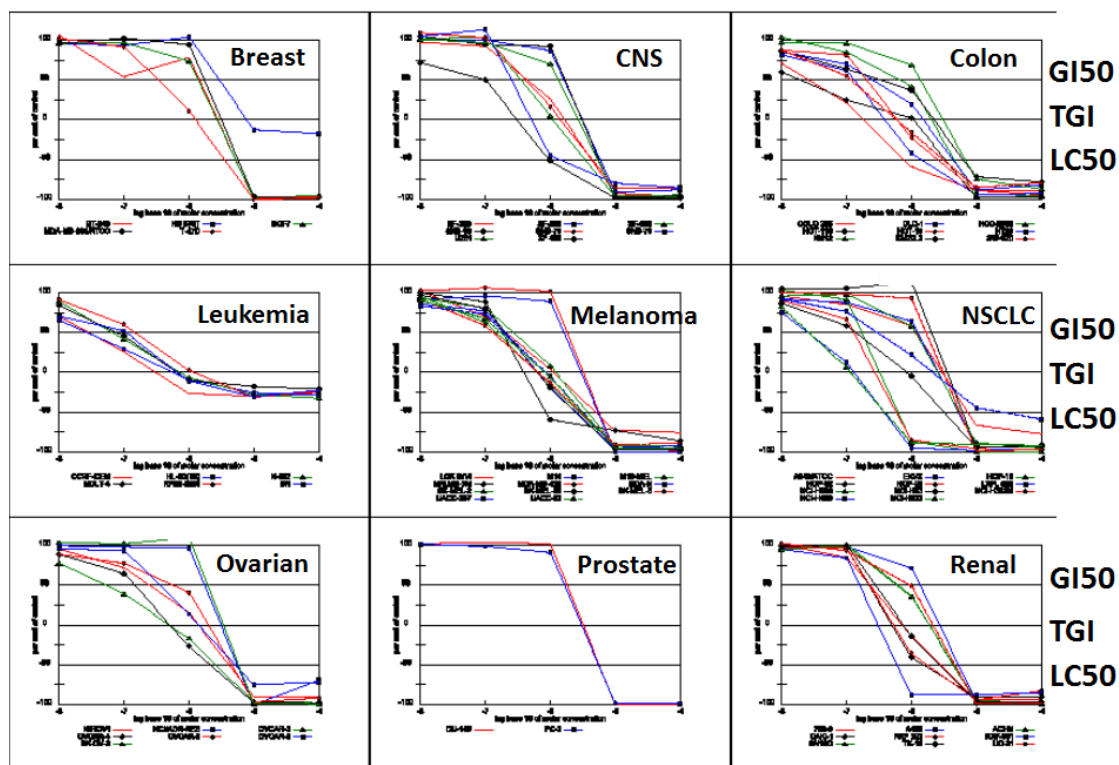


Figure 2. Example of a typical NCI-60 cell line growth inhibition report for compound NSC-625492. Each panel represents cell lines derived from a particular type of cancer (left to right, top to bottom): 5 breast cancer cell lines), 8 central nervous system (CNS) cell lines, 9 colon cancer cell lines, 6 leukaemia cell lines, 11 melanoma cell lines, 11 non-small cell lung cancer (NSCLC) cell lines, 7 ovarian cancer cell lines (including one with P-glycoprotein overexpression), 2 prostate cancer cell lines, 9 renal cancer cell lines). The two small cell lung cancer cell lines are not shown. The compound was tested at the concentration range of 10^{-8} to 10^{-4} M and cells were exposed for 48 hr. Obtained from the NCI DTP database https://dtp.cancer.gov/discovery_development/nci-60/methodology.htm.

In addition to the potency of the drugs (e.g. Figure 2), the selectivity for a certain tumour type *in vitro* (mean graph, Figure 3), the HFA and if available the HTX data, another important selection criterion for the European NCI compounds were the COMPARE data [20]. COMPARE is a comparison tool developed by the NCI based on the sensitivity profile of an NCE compared to the whole database. COMPARE-positive compounds (Pearson correlation coefficient ≥ 0.8) often showed the same mechanism of action; these compounds could be structurally related but also unrelated compounds [9,12]. Since COMPARE-negative compounds (Pearson correlation coefficient < 0.8) usually did not have close matches in the COMPARE programme, they might have a novel mechanism of action and were thought to be most interesting.

For administration of drugs to animals often additional hurdles needed to be solved, such as the administration vehicle, which will be different from formulations used *in vitro* [14]. Many compounds were poorly soluble and specific formulations were used, dependent on the compound, but keeping in mind what might be suitable for future administration to patients. Hence dimethyl sulfoxide (DMSO) might be suitable for *in vitro* and *in vivo* to mice, but not for patients (Table 1). After this pre-formulation step, the potential bioavailability of a compound was investigated. After the development of a suitable HPLC assay (or another analytical assay) and using a proper formulation, the MTD in mice was determined. The MTD was administered and plasma pharmacokinetics determined at various time points to ascertain whether the drug would reach plasma concentrations in the range of the *in vitro* GI50. Subsequently, this dose and schedule were used to explore whether the compound would have suitable *in vivo* antitumor activity. If so,

the development was continued and if not, stopped. In the next section, several examples of such a development will be presented.

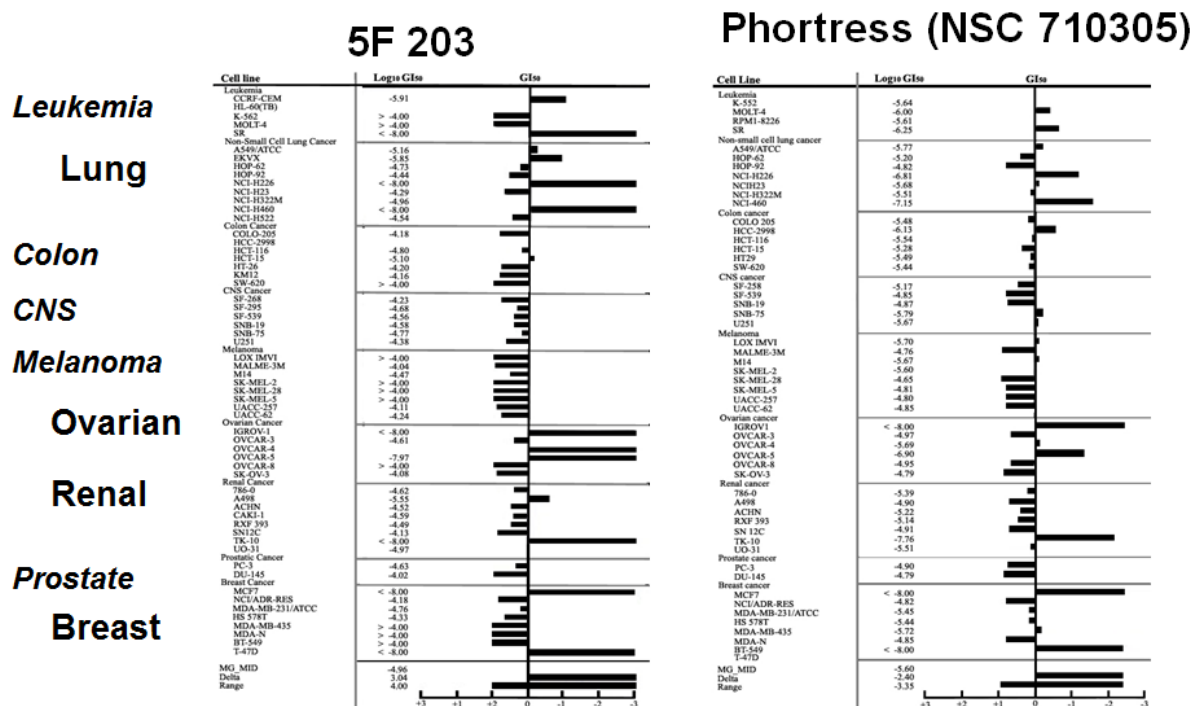


Figure 3. Example report of drug selectivity for 5F-203 and its prodrug Phortress (source NCI DTP). Values are calculated relative to the mean GI50 for each compound. Differences compared to the mean are given as a log 10 value, with bars to left representing less sensitive cell lines (10^3 to 0) and those to the right more sensitive cell lines (0 to 10^{-3}). Cell lines from top to bottom represent 5 leukaemia cell lines, 9 NSCLC, 7 colon cancer, 6 CNS, 8 melanoma, 6 ovarian cancer, 8 renal cancer, 2 prostate cancer, 8 breast cancer (including one with P-glycoprotein overexpression). Please note that the composition of this panel is different from Figure. 2. The most sensitive cell lines are depicted in a larger font. The non-responsive in Italics.

Examples of selected compounds

In one of the early selected series of compounds, NSC-697786 (Figure 4) showed an interesting profile in the COMPARE analysis with a COMPARE coefficient of 0.503 for methotrexate, 0.498 for DUP785 (Brequinar), 0.496 for N-phosphon-acetyl-L-aspartate (PALA) and 0.474 for trimetrexate. This indicates some similarity with antifolates (methotrexate and trimetrexate) and pyrimidine *de novo* nucleotide inhibitors (DUP785 and PALA). The standard approach to exclude this is a comparison in cell lines with aberrations in folate metabolism and to perform rescue experiments with normal pyrimidine nucleosides, respectively. Consequently, this compound was tested in colon cancer WiDr cells grown in standard culture medium (which has a high folate concentration) and WiDr cells that are adapted to a low physiological (comparable to human plasma; WiDr/F) folate concentration [21]. A similar IC50 was found in both lines (0.26 ± 0.06 and 0.25 ± 0.07 μM , respectively; means \pm SE of 4-7 experiments; 72-hour exposure). WiDr/F cells are more sensitive to antifolates such as methotrexate. Addition of 1 or 5 mM uridine or cytidine did not protect the cells, which would have been indicative for a pyrimidine *de novo* inhibition [22]. FMA3 wild type and FMA3/TK- (thymidine kinase deficient) [23] were similarly sensitive (IC50 values for NSC-697786 3.2 ± 0.2 and 2.0 ± 0.8 μM), which excluded a role for thymidine kinase. It was concluded that this compound is not acting as an antimetabolite, and had possibly unique mechanism of action. However, it was discontinued due to lack of *in vivo* activity, possibly because of poor pharmacokinetics.

Table 1. Acceptable solvents for *in vitro* drug solutions or *in vivo* administration of drugs to mice or rats.

Solvent	Maximal concentration	Maximal dose	Species	Maximal final concentration
	<i>In vivo</i>			<i>In vitro</i>
Culture medium				DMEM, RPMI (1 st choice)
water				10% (final)
0.9% Saline pH 7.4		0.5 ml	Mice; rats	2 nd choice
Saline pH 2.0		0.5 ml	Rats (dx5)	Not acceptable
Saline pH 11		0.5 ml	Rats (dx5)	Not acceptable
Ethanol	10%	10 ml/kg	Mice (i.v.)	1%
	10% in water	10 ml/kg	Mice (i.v.; p.o.)	
Dimethylsulfoxide (DMSO)	1% in water	10 ml/kg	Mice (i.p.)	0.25% (0.1% preferable)
	70% in saline	0.2 ml	Rats (dx5)	
Dimethylformamide				0.125 % (upper limit)
	10% in water	10 ml/kg	Mice (i.v.)	
Tween 80	25% in saline	0.2 ml	Rats (dx5)	
	50%	10 ml/kg	Mice (i.v., slow)	
Propylene glycol	25%	10 ml/kg	Mice (i.p.)	
	50%/25%	0.2 ml	Rats (dx5)	
N-methyl glucamine	1% in water	10 ml/kg	Mice (p.o.)	
Carboxymethyl cellulose	1% in water	10 ml/kg	Mice (i.p.)	
Cremophor EL	5% in water	10 ml/kg	Mice (i.v.)	
NaHCO ₃	5% in water	10 ml/kg	Mice (i.v.)	
N,N-Dimethyl-acetamide (DMA)	10% in water	10 ml/kg	Mice (i.p.)	
DMA	10% in arachis oil	10 ml/kg	Mice (i.p.)	

Guidelines developed by the Joint Formulation Working party of the Screening and Pharmacology Group (SPG) which was continued as a PAMM sub-committee and renamed into: Drug Discovery Committee (DDC) [11]

A completely different compound, NSC-625492 was selected for its unique molecular structure being a bismuth analogue (Figure 4). The GI₅₀ was low (0.3 μ M) with activity in many tumour types (colon, ovarian, non-small cell lung cancer and leukaemia; Figure 2). It was thought that it might form DNA adducts, because of its similarity to other heavy metals (e.g. platinum). Using this property, an assay using flameless atom absorption spectroscopy (FAAS) as used for cisplatin [24], was set up to determine its *in vivo* pharmacokinetics. The compound indeed showed interaction with DNA, but was not cross-resistant against cisplatin resistant cell lines and was active in P-glycoprotein expressing cell lines. Using a formulation of 10 % DMA and arachis oil, the MTD was 30 mg/kg (8 % weight loss), which was used to determine its plasma pharmacokinetics (Figure 5) and potential antitumor activity. NSC-625492 did not show a typical bell-shaped plasma profile curve, but the concentrations remained relatively constant over a 24-hr period, possibly indicating a lack of metabolism. Although sufficiently high plasma concentration (> GI₅₀) were found, a long retention and no protein binding (in contrast to cisplatin), there was no *in vivo* activity against

various tumour models. The development of the compound was therefore discontinued.

NSC-637828 also showed an interesting COMPARE negative profile (correlation coefficients < 0.625) with rifamycin and the nitrogen mustard CCNU (Lomustine; 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) being the highest. It was insoluble and a suspension could only be obtained in 10 % DMA + arachis oil (but not in DMSO or ethanol), which showed acceptable toxicity (< 10 %). Due to these properties an adequate HPLC method could not be established and drug sensitivity studies could not be completed. Therefore, the development of this compound was stopped.

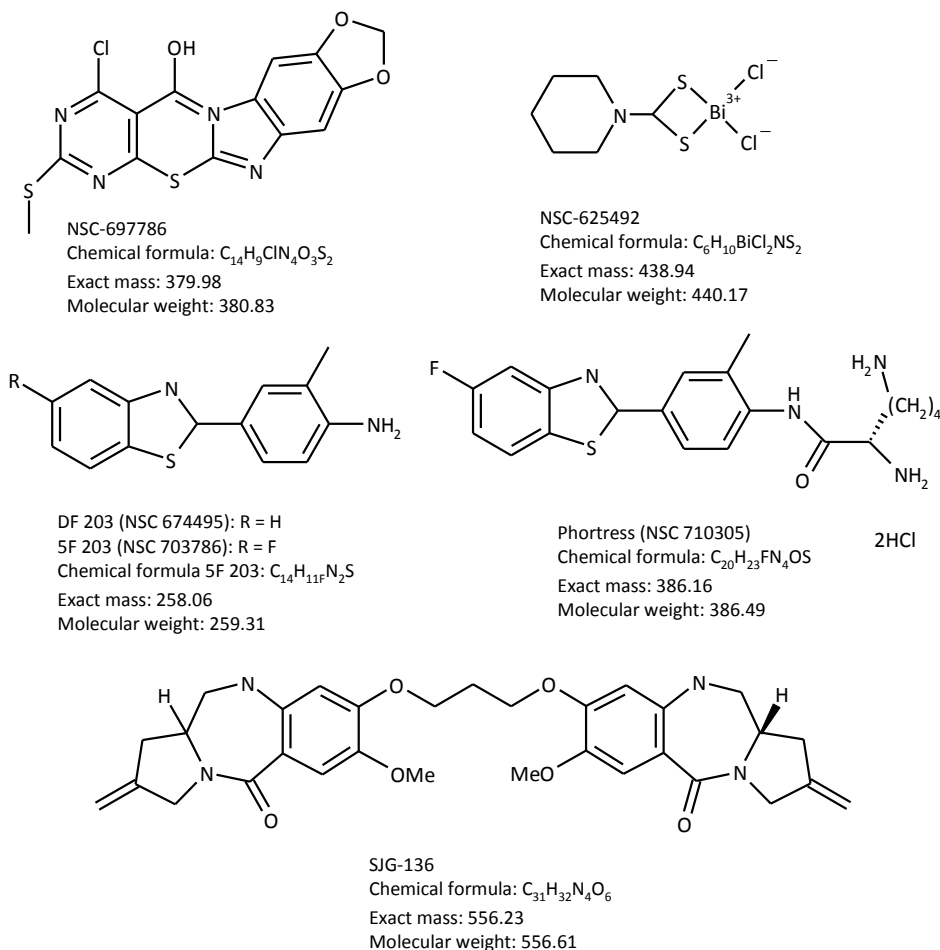


Figure 4. Structural formulas and some physico-chemical properties of several of the investigated compounds.

Two examples of drugs with completely different mechanisms of action are the combretastatin A-4 analogues and NSC 734237, a Paullone-like molecule. These two drugs were investigated in a collaborative effort of various PAMM laboratories and the NCI Drug Development and Target Analysis Branches. Combretastatin A-4 is a poorly soluble natural product and its more soluble metabolite combretastatin A-4 phosphate disrupts endothelial junctions of tumour blood vessels by preventing microtubule polymerisation in endothelial cells of tumour blood vessels; this resulted in tumour necrosis [25,26]. Various analogues (NSC736359, NSC736992, and NSC733436) with a high potency (nM- μ M range) were further analysed, but moderate antitumor activity was observed [27], possibly because under these conditions the density (size and number) of CD31⁺ blood vessels was not affected.

The Paullone-like molecule NSC 734237 is an isoindoloquinoline with low to sub μ M IC₅₀ values [28] for CNS, colon, melanoma and breast cancer lines. However, the compound was quite insoluble (6 % active drug in a liposomal formulation), but showed antitumor activity against a doxorubicin-resistant, oestrogen

receptor negative human breast cancer xenograft MT-3/ADR with a reduction in both CD31⁺ microvessel density and Ki-67. Hence the drug showed antivascular effects and cytotoxic activity, which are most likely due to inhibition of tubulin polymerisation and topoisomerase I, respectively. More water soluble analogues were investigated further, but did not improve the overall result. In the future, such compounds should optimally be investigated more extensively for their oral bioavailability and their formulation adapted.

Concentration-time curve of NSC-625492 plasma of mice: total and free bismuth

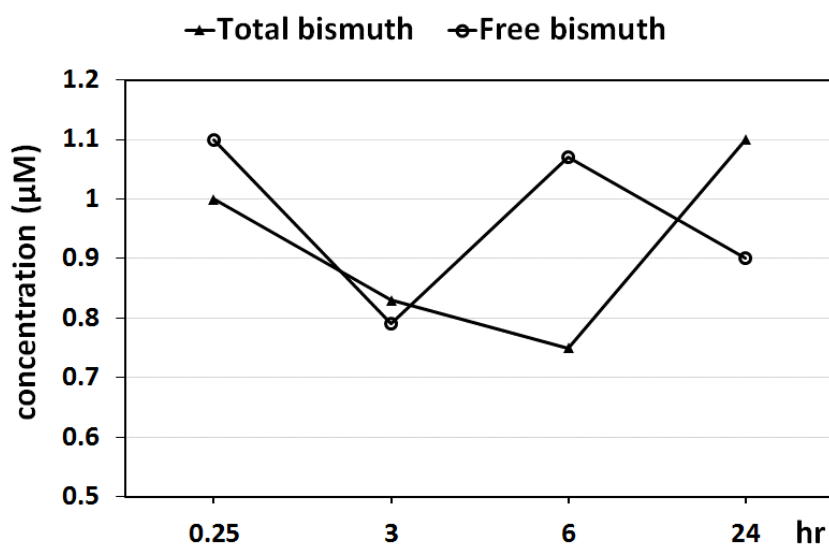


Figure 5. Concentration time curves of NSC-625492. Mice were injected with 30 mg/kg NSC-625492 in 10% DMA in arachis oil. For the pharmacokinetics study mice were sacrificed at 0.25, 3, 6 and 24 h and blood was collected by heparinization to prepare plasma. Total bismuth was measured by mixing 1 volume of plasma with 5 volumes of triton/antifoam (1/1) and 4 volumes of 0.4 M HCl, which was directly introduced into the AAS (Varian SpectrAA 300 Zeeman with certified Bi-lamp; $\lambda=306.8$ nm). Calibration lines were obtained with bismuth nitrate from 0.17 to 3 μM . Free bismuth (non-protein bound) was measured in the supernatant after deproteinization (1 volume of plasma mixed with 2 volumes of ethanol and put at -20 °C for 2 hr followed by centrifugation for 10 min at 4000 g)

However, several compounds that showed an interesting profile in the NCI-screen, were also successful at further evaluation, such as DRH-147 and Phortress. DRH-417, a pyrrolobenzodiazepine monomer acts by binding into the minor groove of DNA and was very potent in the NCI 60-panel (mean IC₅₀ 3 nM) and at subsequent testing by investigators of the European NCI compounds group [29]. It showed favourable pharmacokinetics (plasma peak levels of 171 nM, far above the GI₅₀ value) and interesting *in vivo* activity against several HTX models ($p<0.01$). These data led to the development of SJG-136, a pyrrolobenzodiazepine dimer that went into a clinical Phase I study and Phase II studies in ovarian cancer and leukaemia [30].

Phortress (Figure 3) is a water-soluble benzothiazole prodrug of 5F-203, that is active (low nanomolar GI₅₀), and selective against breast, ovarian and lung cancer [31]. 5F-203 binds to cytoplasmic aryl hydrocarbon receptors, forming a complex that is translocated to the cellular nucleus, and subsequently binds to the *cyp1a1* promoter [32,33]. This leads to an induction of the expression of cytochrome P450 1A1, responsible for metabolism of 5F-203 to a bio-reactive species that forms DNA-adducts. This unique mechanism of action, together with a good pharmacokinetic profile, led to further evaluation in Phase I clinical study [34], in which 14 out of 42 patients had stable disease. All together, these data show that the approach can lead to compounds with clinical activity.

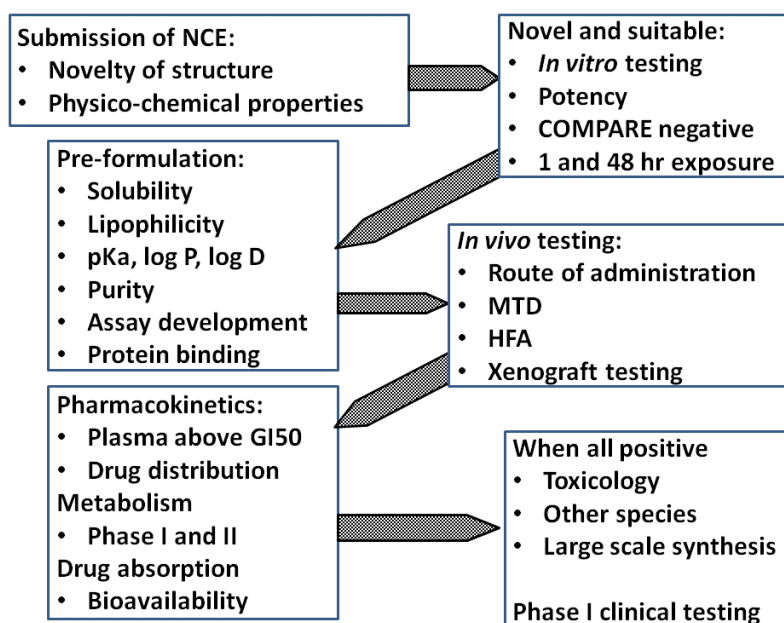


Figure 6. Flow chart showing the testing procedure and addition of novel parameters as outlined in the text. Each step can lead to elimination when not full-filling the criteria. NCE, new chemical entity; MTD, maximal tolerated dose; HFA, hollow fiber assay.

The first three examples show that despite initial enthusiasm about the drug sensitivity profile and COMPARE data, development had to be discontinued rapidly. Unfavourable physico-chemical properties, such as insolubility, made it impossible to perform adequate *in vitro* and *in vivo* studies and to set up suitable analytical assays. In addition to this and lack of *in vivo* activity, other reasons for discontinuation included instability, impurity, unavailability of material and insufficient bioavailability. A careful analysis of the mechanism of action helps to understand the success (DRH-147 and Phortress) but also enabled a systematic development of novel analogues. This was due to a successful scientific collaboration between different groups that provides a model for future drug development. Importantly the groups adhered to standard cellular pharmacology tools for testing [14].

Conclusions

The results of the pharmacologically directed approach clearly showed that a few simple procedures with small numbers of mice quickly allow discontinuing agents that were highly unlikely to have any antitumor activity. The process is outlined in the flow chart (Figure 6). The chart also shows some streamlining to optimize the process and eliminate agents with unsuitable pharmacological parameters in less than three months. Current approaches should put much more emphasis on physico-chemical properties such as solubility, *pKa* values, *log P* and *log D* values, protein binding and drug distribution. This serves two purposes, whether to discontinue development rapidly or when promising how to design an analogue with better properties. Regarding solubility, there are novel possibilities to use insoluble compounds with adequate solvents [35]. Using biomimetic approaches one would be able to gain insight in elimination constants, and likelihood of brain uptake [36]. Next to that, pharmacological evaluation should include Phase I (oxidation) and II (conjugation) drug metabolism which can currently easily being studied using isolated hepatocytes. Inclusion of such novel approaches would more rapidly predict whether a drug will have suitable pharmacological properties to proceed to the next step in development.

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