Issues in experimental design and endpoint analysis in the study of experimental cytotoxic agents *in vivo* in breast cancer and other models

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Summary

Considerable effort has been placed into the identification of new antineoplastic agents to treat breast cancer and other malignant diseases. The basic approaches, in terms of model selection, endpoints, and data analysis, have changed in the previous few decades. This article deals with many of the issues associated with designing *in vivo* studies to investigate the activity of experimental and established compounds and their potential interactions. Endpoints for both *in situ* and excision assays are described, including approaches for determining cell kill, tumor growth delay, survival, and other estimates of activity. Suggestions for approaches that may limit the number of animals also are included, as are possible alternatives for death as an experimental endpoint. Other concerns, such routes for drug administration, drug dosage, and preliminary assessments of toxicity also are addressed. Statistical considerations are only briefly discussed, since these are addressed in detail in the accompanying article by Hanfelt (Hanfelt JJ, Breast Cancer Res Treat 46:279-302, 1997). The approaches suggested within this article are presented to draw attention to many of the key issues in experimental design and are not intended to exclude other approaches.

Introduction

The disseminated nature of breast cancer and the development of crossresistant tumors are the primary causes of failure of current therapies. By the time many tumors are detected, there is a high probability that metastatic lesions will be present, many of which may already contain resistant subpopulations [1]. Not surprisingly, there is substantial interest in the identification of novel cytotoxic and endocrine agents for the treatment of breast cancer.

A systemic approach is required to eradicate the majority of metastatic breast disease, and this remains primarily in the form of cytotoxic chemotherapy or endocrine manipulation. The latter began with the initial studies on oophorectomy by Beatson [2] and was followed by the administration of high dose estrogens. These were largely replaced by the development of antiestrogens,

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Table 1. Several of the human breast tumor cell lines used as xenografts in the current NCI drug screening program*

Cell Line	ER status	Citation
BT-549	negative	**
Hs578T	negative	(106)
MCF-7	positive	(107)
MCF7/ADR (multidrug resistant)	negative	(39,40,106)
MDA-MB-231 (metastatic)	negative	(108,109)
MDA-MB-435 (metastatic)	negative	(108-110)
T47D	positive	(111)

* Kindly provided by Dr. Joseph Mayo.
** There is no primary citation for this cell line listed by the provider (ATCC, Rockville, MD), who indicate in their "Catalogue of Cell Lines and Hybridomas" that the cells were derived from a papillary invasive ductal tumor in a 72-year old patient. Metastatic disease was found in three of seven regional lymph nodes. The originators were Drs. W.G. Coutinho and E.Y. Lasfargues. A discussion of other human breast cancer cell lines can be found elsewhere [112].

initially the triphenylethylene Tamoxifen [3] and, more recently, introduction of the steroidal anti-estrogen ICI 182,780 [4]. Other endocrine strategies include the use of inhibitors of estrogen biosynthesis, primarily aromatase inhibitors, and the use of LHRH agonists and antagonists.

The use of chemotherapeutic agents in the management of neoplastic disease began with Rhoad's description of the use of nitrogen mustard for the treatment of Hodgkin's lymphoma [5]. The number of cytotoxic agents available has increased substantially over the intervening years, with breast tumors generally exhibiting good overall response rates to several cytotoxic drug combinations. While chemotherapy can produce gains in overall survival, most patients with metastatic breast cancer will eventually recur. Many reasons may account for this failure, including poor dose scheduling, inappropriate combinations of drugs and the emergence of cell populations resistant to the antineoplastic agents.

Animal models provide one approach for the optimization of drug scheduling and for the identification of novel compounds that exhibit promise in such *in vitro* prescreens as those currently utilized by the National Cancer Institute [6]. For many years a primary *in vivo* screen, utilizing the L1210 and P388 murine leukemias, was an integral part of the NCI's preclinical drug development program. However, the lack of a significant representation of solid human tumors drew criticism, and partly explained the weakness of the screen to identify new agents active against the more common solid tumors, including breast cancer. Perhaps not surprisingly, the screen appears to have been more successful in identifying agents active against hematologic malignancies. Some drugs with well established clinical efficacy (busulfan, hexamethylmelamine) fail to demonstrate substantial activity in the L1210/P388 screen [7,8].

The P388 and L1210 *in vivo* screen has largely been replaced by panels of disease specific human tumor cell lines (prescreen) and xenografts (primary screen). While there are relatively few ideal *in vivo* models for breast cancer, several of the available human cell lines fulfil some of the requirements for screening, *e.g.,* stable phenotype, high tumor take rate, predictable and reproducible kinetic properties. Several of those currently used by NCI are shown in Table 1.

Some considerations for choice of model, scheduling, dosage, and endpoint will be discussed below. The discussions and issues raised are to draw attention to different approaches to experimental design. As such, these should be considered in the light of the other articles on experimental design and data analysis in this issue and elsewhere. Some issues are generic, while others are more directly applicable to screening cytotoxic activities, and may be of lesser relevance to testing chemopreventive and endocrine agents. It is hoped that the topics discussed will assist investigators to consider key issues in experimental design. However, these are provided only as suggestions. There are many ways to identify potentially active compounds and drug combinations, and these are constantly being

modified by improvements in both cellular models and approaches to the use of animals in biomedical research.

Log cell kill and tumor kinetics in cytotoxic cancer chemotherapy

Early attempts to modify cytotoxic therapies were largely empirical. The initial criteria for designing cytotoxic therapies were based on the observations of Skipper, who demonstrated that cytotoxic drug-induced cell kill follows a similar kinetic pattern to that established by Arrhenius at the turn of this century for the killing of bacteria. The log cell kill hypothesis states that cytotoxic drugs kill cells by first order kinetics [9]. Thus, a constant proportion of the cells will be killed regardless of the size of the cell population. For many cytotoxic drugs a 4-log cell kill is achievable and would eradicate a tumor population of 10^3 cells and have a one in ten chance of eliminating a population of 10^4 cells [10]. However, clinically detectable primary tumors have a cell population frequently in excess of 10^9 cells. Many metastases also could contain cell populations greater than 10^4 cells. While theoretically sound, log cell kill can be affected by several biological parameters, including the presence of *de novo* resistant cells, the degree of tumor vascularity, and other factors that may affect drug perfusion and metabolism.

The principles elucidated by Skipper were further modified by Norton & Simon, who applied Gompertzian growth kinetics to tumor cell populations [11]. This clarified the inverse relationship between growth fraction and tumor size. The ability of a cytotoxic drug to inhibit tumor cell growth was determined to be directly related to the tumor's growth rate, which also is related to tumor volume [12]. The tumor mass killed by a cytotoxic treatment is proportional to the growth fraction multiplied by the total tumor volume [12].

A Gompertzian kinetic growth pattern produces a growth fraction that ultimately decreases exponentially with time. This inverse relationship between tumor size and growth fraction implies that micrometastases should be more kinetically sensitive to cytotoxic chemotherapy than larger tumor masses [13,14]. Thus, early intervention when the tumor mass is small should provide the greatest opportunity for induction of remission. Metastases tend to exhibit a more rapid tumor doubling time (T_D) than primary tumors, particularly for the common solid tumors like those of the breast [15].

There are a number of factors that contribute to the apparent T_D of any tumor. These include the rate of cell production, the size of the growth fraction, cell recruitment from G_0, and the rate of cell loss. Cell loss includes shedding of cells into other compartments, *e.g.*, metastasis, differentiation to a non-proliferating cell type, or entry into prolonged G_0 and apoptotic cell death. The high proportion of non-proliferating or normal cells present in many solid tumors, and the frequently high rate of cell loss, generally produce tumors with a long T_D. The rate of cell loss may be as high as 80% of the rate of cell production [16].

Growth kinetics in human tumors and animal models

The kinetic parameters of tumor growth represent one of the major differences between animal models and the human disease. While Gompertzian kinetics apply to experimental tumors and those in patients, the T_Ds and growth fractions are frequently quite different. For example, many human breast tumors exhibit long T_Ds and often small relative growth fractions. In marked contrast, human breast tumor xenografts generally have short T_Ds and high growth fractions. Estrogen-treated MCF-7 tumors (ER-positive) have T_Ds of approximately 10-12 days [17-19] compared with over 100 days for many tumors in patients [20]. We have generated several estrogen-independent MCF-7 variants. These have T_Ds as long as 100 days when grown in the absence of

estrogen, but grow as rapidly as parental MCF-7 tumors in estrogen-supplemented mice [17-19]. MDA435/LCC6 cells (ER-negative, ascites variant of MDA-MB-435) growing as solid tumors have much shorter T_Ds of 3-5 days [21]. Most of the breast cancer xenografts used in the current NCI screen have mean T_Ds of 2-10 days.

The differences in kinetic properties between xenografts and tumors in patients would tend to make the xenografts more sensitive to agents with a strong cell cycle/cell phase specificity. For the purposes of a primary *in vivo* screen for novel agents, a limited overestimate of activity may not be a major concern. For studies to optimize scheduling or combinations of established drugs, the relative sensitivity of the *in vivo* screen is a concern only if the model is either too sensitive or too resistant to a combination, when it will become difficult to assess interactions. Most of these concerns are readily addressed by a careful choice of *in vivo* model(s). Various schedules have already been identified for the established drugs; examples are provided in Table 2.

The dose response relationship and dose intensity

The relationship between treatment and response is described by:

$$k = C \times t$$

where C = concentration; t = time. Thus, response should be approximately equivalent where C x t values (area under the concentration time curve) are equivalent. This can enable the design of clinically relevant *in vitro* analyses of established drug combinations based on pharmacokinetic measurements previously obtained in patients or animals. Clinical studies can utilize the reasonable across-species dosage relationship of mg/m^2 to estimate dose from data obtained in preclinical animal screening. The doses may require some further modification, since the serum half-life of some drugs can be longer in man than in rodents [22]. One approach is to use 1/10th

the maximum tolerated dose (MTD) in rodents as the approximate starting dose for a Phase I trial in humans [23]. Where possible, it may be better to use a dose that produces comparable pharmacokinetics, since this can increase the predictability of the mouse xenograft-to-human tumor model [24].

The pharmacokinetics for cytotoxic drugs are frequently similar in mice and men [25]. For many drugs, the mouse LD_{10} also approximates the MTD in humans when expressed as mg/m^2 [26]. However, there are exceptions. The C x t values at the LD_{10} are higher for mitomycin C, vincristine, and cyclophosphamide, and lower for methotrexate and 5-fluorouracil, in mice when compared with humans (reviewed in [26]).

In clinical practice, a narrow therapeutic index is frequently responsible for the reduction of dosage due to side effects. However, the steep dose response curve for most cytotoxic drugs implies that even a small perturbation in dosage may produce a significant change in response. The effects of alterations in dosage on clinical response has been widely reviewed [22,27]. It has been suggested that a major contributing factor to the failure of many treatments is the *ad hoc* reduction of drug dosage [22].

It has been widely acknowledged that the most effective treatments involve a high dose intensity chemotherapeutic regimen. This is partly based on the steep dose response relationship for most cytotoxic drugs and various other clinical observations. Dodwell *et al.* [27] have reviewed the published data, and reexamined the role of dose intensity in response, for a number of the more common malignancies. While they conclude that high intensity regimens can produce significant advantages in disease free survival, clear demonstrations of increased overall survival are obtained much less frequently. The relationship between dose intensity and response often varies with both drug, tumor model, and disease. Animal models provide a safe and logical means to explore this and related issues, rather than attempting to identify appropriate or potentially dangerous schedules directly in patients.

Table 2. Examples of dosages and schedules for several established cytotoxic agents. The tumor models against which the drugs were tested were of various origins, and not exclusively breast. Some drugs that are not widely used in breast cancer are included, since these may be useful as controls for establishing the extent to which a tumor model reflects the human disease. Toxicity can vary with strain, sex, age and other parameters, and the information in this Table reflects that diversity.

Drug	Dose[1]	Route	Schedule[2]	Toxicity	Citation
Adriamycin	6 mg/kg	i.v.	4,6,8	ND[3]	[67,113]
	6 mg/kg	i.v.	1,5,9	ND	[114]
	6.8 mg/kg	i.p.	single dose	none[4]	[21]
	8.5 mg/kg	i.p.	single dose	LD[5]	[21]
	24 mg/m^2	i.v.	single dose	MTD	[25]
Ara-C[6]	40 mg/kg	s.c.	24 hr infusion	none	[115]
	50 mg/kg	i.p.	1-7	alopecia	[116]
BCNU	18 mg/kg	i.p.	single dose	LD	[21]
Methyl-CCNU	18 mg/kg	i.p.	single dose	MTD[7]	[71]
Cyclophosphamide	35 mg/kg	i.p.	single dose	alopecia	[116]
	60 mg/kg	i.p.	0-4, 7-11	ND	[117]
	100 mg/kg	i.v.	1,5,9	ND	[114]
	143 mg/kg	i.p.	single dose	none	[118]
	200 mg/kg	i.p.	1,15	LD	[119]
	286 mg/kg	i.p.	single dose	LD	[118]
	290 mg/kg	i.p.	single dose	none/LD	[81]
5-Fluorouracil	32 mg/kg	i.p.	5,6,7,8	ND	[72]
	40 mg/kg	i.p.	1-4, 15-18	LD	[119]
	50 mg/kg	i.p.	0-4	ND	[117]
	60 mg/kg	i.v.	1,5,9	ND	[114]
	180 mg/m^2	i.p.	single dose	MTD	[25]
Ifosfamide	150 mg/kg	i.v.	single dose	none	[77]
	300 mg/kg	i.v.	single dose	none	[77]
Melphalan	12 mg/kg	i.p.	single	ND	[120]
	12 mg/kg	i.p.	0,4	ND	[120]
Methotrexate	4 mg/kg	i.p.	1,4,8,11,15,18	LD	[119]
	15 mg/kg	i.v.	1-5	ND	[119]
	20 mg/kg	i.p.	0-3	ND	[117]
Mitomycin C	2 mg/kg	i.v.	1,15	LD	[119]
	2.5 mg/kg	i.v.	single dose	LD	[121]
	4.5 mg/kg	i.p.	single dose	none	[21]
	5 mg/kg	i.p.	single dose	ND	[122]
	18 mg/m^2	i.p.	single dose	MTD	[25]
Cis-Platinum	4 mg/kg	i.p.	1,5,10	ND	[123]
	4 mg/kg	i.v.	1,5,10	ND	[123]
	7.5 mg/kg	i.p.	single dose	none	[21]
	10 mg/kg	i.p.	single dose	ND	[123]
	27 mg/m^2	i.p.	single dose	MTD	[25]
Taxol	20 mg/kg	i.p.	single dose	none	[21]
Taxotere	15 mg/kg	i.v.	4,6,8	MTD	[67]
Vinblastine	3 mg/kg	i.v.	1,15	LD	[119]

1 = Dose/injection; 2 = Schedule is given as days unless otherwise indicated; 3 = Not defined or not described; 4 = None reported or no deaths; 5 = Lethal dose (one or more deaths attributed to drug-induced toxicity); 6 = 1-β-D-arabinofuranosylcytosine; 7 = Approximate MTD as defined by the investigators.

General considerations in experimental design

Choice of host for xenografts

There are several considerations relating to the choice of immunocompromised host, including the degree of immune-competence and the ability of the strain to support the growth of the tumor. For syngeneic tumors the choice of host should be obvious. However, the choice of host for xenografts is more complex. The host not only should facilitate the growth of the tumor but also should enable the tumor to maintain a biologically relevant phenotype. It also is useful if the selected model enables some estimation of the selectivity of drug action and the determination of potentially lethal toxicities. For most of the breast cancer cell lines/xenografts available, the *nu/nu* mouse is sufficient, irrespective of the background strain [28].

The potential for immunological modulation to contribute to tumor response to combination chemotherapy is an important consideration. This is particularly relevant when the combination includes biological response modifiers that can influence the activation of cells associated with cell mediated immunity, *e.g.,* interferons and interleukins. In some cases, it may be desirable to attempt to either eliminate or exclude effects on the immune competence of the host. Thus, the choice of an appropriate immune-deficient strain may become paramount. Since the different immunobiologies of available rodent hosts have been recently reviewed [28], they will not be further discussed.

Choice of appropriate tumor model

The model used for the screening of a drug suspected of activity against a particular tumor should reflect the biological properties of that tumor as closely as can reasonably be achieved. For example, a drug active against a leukemia with a short T_D, high growth fraction, and relatively short cell cycle time would be less likely to demonstrate activity in a screen against a breast tumor with a longer T_D, small growth fraction, and long cell cycle time. Thus, choice of an appropriate model to screen combinations of agents with known pharmacological and kinetic requirements should (where possible) closely reflect the major biological properties of the human disease. To some extent the model also should reflect the requirements of the drug. For example, there would be limited value assessing a drug expected to show no crossresistance to P-glycoprotein without including a model that expresses P-glycoprotein.

Selecting a breast tumor model for screening cytotoxic compounds can be problematic. Relative to the murine ascites models, most solid breast tumors exhibit a relatively slow T_D. For example, MCF-7 tumors have a T_D of approximately 10-12 days when growing in appropriately estrogen supplemented animals [19]. While this may be acceptable for screening antiestrogenic or chemopreventive agents, estrogenic supplementation can alter the activity of some cytotoxic drugs [29,30]. In general, the most rapidly proliferating human breast tumor xenografts do not express estrogen receptors. MDA435/LCC6 tumors have T_Ds of 2-3 days. Since the parental cell line (MDA-MB-435) was obtained from a patient who had not received chemotherapy, these models may be useful in screening cytotoxic agents for activity against breast cancer [21]. The MDA-MB-231 cell line also has a comparably rapid T_D *in vivo* (R. Clarke, unpublished observations). The more rapid T_Ds of ER-negative xenografts broadly reflect the characteristics of such tumors in patients, where ER-negative tumors tend to have shorter T_Ds [31].

From a purely practical viewpoint, relatively slowly proliferating breast tumors can produce significant problems in logistics and experimental design. The slower growing tumors frequently exhibit significant intertumor variability and can require substantial numbers of animals to enable meaningful statistical analysis of data. Determining the period at which a tumor is considered

"cured" also can become problematic. Even the more rapidly proliferating solid tumors with T_D = 48 hr may require up to four months of post treatment observation to establish "cure" [32].

It is unlikely that any one tumor model will adequately represent the major biological characteristics of a particular malignancy. Thus, the use of a series of tumors (where appropriate/available) may be required to determine the sensitivity of a particular neoplastic disease to a either a single or a combination chemotherapy regimen. However, this must be considered in the context of reducing animal usage, cost, and the value of the additional data obtained.

For breast cancer, there are several potential models available for screening (Table 1). Most of the ER-positive models require estrogenic supplementation for tumorigenicity or maximal growth. We have generated ER-positive models that will grow without supplementation, but the respective T_Ds are relatively long [19]. While this may be more representative of breast tumors in general, this characteristic is inappropriate for screening cell cycle or cell phase specific cytotoxic compounds. We also have developed an ascites model based on a variant of the MDA-MB-435 cell line (MDA435/LCC6). The pattern of response to a variety of cytotoxic drugs appears to reflect closely that seen in breast cancer patients [21]. For example, breast cancers in general respond poorly to nitrosoureas [33], as do MDA435/LCC6 ascites to BCNU. Etoposide also does not produce long term survivors, and this drug generally has been ineffective as a single agent in breast cancer [34,35]. Adriamycin [34], mitomycin C [36], and taxol [37] are among the most effective single agents in previously untreated breast cancer, and all of these drugs produced long term survivors in mice bearing the MDA435/LCC6 ascites. The characteristics of several breast cancer xenografts have been reviewed elsewhere [28].

Another example of the choice of tumor model is in studies to evaluate P-glycoprotein reversing agents, compounds which may have significant potential in some breast cancer patients [38]. For these types of analyses the choice of tumor model is critical. Cells to be used as xenografts should be transfectants rather than selected for resistance, since selection can produce multiple unrelated resistance mechanisms. For example, MCF-7^{ADR} (selected for resistance to adriamycin [39]), but not MDR1-transduced MCF-7 cells (CL 10.3), are cross resistant to Tumor Necrosis Factor [40]. Since both adriamycin and Tumor Necrosis Factor can inhibit cells by the generation of free radicals [41,42], this cross resistance in MCF7^{ADR} cells suggests the presence of functional adriamycin resistance mechanisms in addition to P-glycoprotein, including altered expression of manganous superoxide dismutase [40]. These cells also exhibit increased glutathione transferase and topoisomerase II activities [43,44]. The use of transfected cells allows for a clearer interpretation of the data.

Cells concurrently expressing multiple resistance mechanisms may more closely reflect the drug resistance that occurs in patients [38], but interpreting responses in a mechanistic light may be difficult. This does not invalidate their use where the purpose is simply to screen compounds for potential antineoplastic activity. Indeed, the choice of a series of models that are too sensitive will likely identify compounds with limited activity in patients, whereas active compounds identified in otherwise resistant models may have a higher probability of being active in patients [45]. This is likely to be true in principle, but the extent to which it applies will depend upon whether the resistance mechanisms operating in the tumor model contribute significantly to the resistance phenotype in patients.

It is apparent that there are two potential types of screening approaches, each with different objectives that will result in different choices of models. Where a broad based, non-mechanism oriented screen is required, a disease-specific panel of xenografts with widely differing but biologically relevant phenotypes is likely to be optimal. Knowledge of the pattern of response to a series of established drugs, for each component of the panel, is required. Such a panel might be

expected to contain both sensitive and resistant models (see Table 1 for examples included in one possible panel for breast cancer). For a mechanism or structure/function based screen, the choice of components is likely to depend upon assumptions inherent in the mechanism. For example, where a specific target is identified, the panel may contain several models with different levels of expression of the target, *e.g.,* P-glycoprotein, multidrug resistance related protein [21,38,46,47]. In many cases, this requirement may be most effectively met by a series of transfected cell lines and their respective control populations.

Phenotypic stability

The stability of the phenotype is a critical determinant for tumor model selection. Some tumor xenografts may require periodic cycles of *in vivo/ in vitro* growth in order to maintain the ease of reestablishment *in vitro* for some excision cytotoxicity assays. Prolonged *in vivo* growth of some established cell lines can result in significant phenotypic alterations. We have described the isolation of hormone-independent sublines of the estrogen-dependent MCF-7 human breast cancer cell line following prolonged selection *in vivo* [17,18]. While responses to antiestrogens remain unaltered [18,48], there are significant changes in their responsiveness to estrogens [17,18,48]. For many cell lines, this problem can be overcome by using cells within a limited number of passages (≤10) from a single frozen stock of cells. The frozen stock should be from a single passage of cells with a well characterized phenotype.

The intertumor stability of the growth patterns and cell cycle profiles also may be important considerations. A high degree of variability in intertumor growth fractions could significantly influence the reliability or ease of data interpretation. The stability of the metastatic potential must be well defined. Cells with an unpredictable metastatic capacity may alter tumor burden and affect survival and/or the host's sensitivity to the toxicity of cytotoxic treatments.

Therapeutic index and dose scheduling

The difference in the dose response curves of normal and neoplastic tissues, often referred to as the therapeutic window or therapeutic index, is widely applied in the clinical pharmacology of cytotoxic drugs [22,49-51]. This difference in drug sensitivity enables the administration of sufficient drug to produce cytotoxic effects in the tumor, but not to induce significant and irreversible toxicity to normal cells. Unfortunately, many cytotoxic drugs exhibit a steep dose response curve with a small therapeutic index. In many cases, the development of unacceptable toxicity is the dose limiting factor for cytotoxic chemotherapy.

The sensitivity of normal cell populations reflects the rapid growth rate and high growth fraction of some cells. For example, cells in normal bone marrow and the intestinal crypts have respective thymidine labeling indices of 30%-70% and 12%-18% [15]. These values are generally higher than observed in most solid tumor cell populations. This accounts for the high incidence of hematopoietic and gastrointestinal side effects associated with many chemotherapeutic regimens. Granulocytes are highly susceptible to cytotoxic agents because of their high growth fraction and short lifespan, but a pool of non-cycling stem cells ensure repopulation [15]. Generally, the purpose of dose scheduling is to administer the second and subsequent treatments at times that will allow some normal stem cells to evade the drug and enable repopulation to occur without permanent damage to bone marrow. Examples of some drug schedules for rodents are provided in Table 2.

Drug administration in vivo

The route of administration can influence drug pharmacokinetics, toxicity, and antitumor activity. For example, injection *i.v.* introduces the drug directly into the blood, with a relatively rapid exposure of hematopoietic stem cells. Adminis-

tration *s.c.* or *i.p.* would be expected to produce a slower drug equilibration with this compartment, and produce a delayed or reduced myelosuppression. However, local tissue damage could be increased relative to the *i.v.* route. The potential toxic/pharmacokinetic differences associated with routes of administration can be controlled by careful planning. For example, while an *i.v.* bolus of an agent may produce unacceptable toxicity, the same dose in mg/kg body weight can occasionally be administered either by infusion, *s.c.,. p.o.,* or multiple lower doses given *i.v.,* with significant alterations in host toxicity.

For the majority of solid tumors, an *i.v.* administration most closely reflects the clinical administration of the drug. However, for experimental drugs, the route of administration will vary primarily with the physico-chemical properties of the drug. The *i.v.* route is generally limited to water soluble compounds with a pH >4.0 and <8.5. Water-insoluble drugs can be administered either *s.c.,* provided they do not produce unacceptable local damage, or *p.o.* if they have sufficient chemical stability. For steroid hormones and antihormones that can require sustained delivery, *i.p.* or *s.c.* depots in peanut oil, *s.c.* Alzet mini pumps (Alza Scientific, Palo Alto CA), cholesterol-based slow release pellets (Innovative Research of America, Sarasota, FL) or silastic pellets all can produce appropriate plasma levels of drug for sustained periods of time.

The timing of administration also is an important consideration. Initiation of treatment within a few days of tumor cell inoculation may produce evidence of activity, when administration to established tumors indicates inactivity. For cytotoxic agents, administration within a few days of cell inoculation is often inappropriate. Treatment of established tumors, where these are clearly palpable and fall within a predefined size range, is generally more appropriate and allows for assessment of the most widely used endpoints. This size should not be so large as to influence response to the drug. Early administration of drug is usually appropriate when the tumor is

directly xenografted from another animal and has a rapid T_D of only a few days, or perhaps when sensitivity is required in a primary screen. Another exception is for the testing of chemopreventive agents, *e.g.* antiestrogens or retinoids, which could be given to high risk women without evidence of clinically detectable disease. With these compounds, chemopreventive/chemosuppressive activity against low tumor burdens, *e.g.* recently inoculated tumor cell suspensions, may more closely reflect the proposed clinical use. Where initiation of treatment around the time of tumor cell inoculation is justified, *i.e.* before the appearance of palpable tumors, the more common endpoints include time to tumor appearance and tumor incidence.

Pharmacokinetics and toxicity in drug combination studies

There are a number of pharmacokinetic considerations that can influence activity of a drug *in vivo* when drugs are combined. The biological activity of one agent may alter the metabolism, absorption, distribution, or toxicity of another. Insulin can alter the metabolism and subsequent cytotoxicity of methotrexate [52]. Prednisolone reduces host toxicity and enhances the antitumor effects of nitrogen mustard, melphalan, and chlorambucil [53]. In some experimental tumor systems progesterone can reduce the systemic toxicity of chlorambucil [53]. Martin *et al.* [54] have reported that adriamycin, lomustine, carmustine, semustine, and vincristine can increase melphalan uptake in L1210 cells. In patients with advanced breast cancer, the toxicity of a combination of cyclophosphamide, methotrexate, and 5-fluorouracil is reduced by fluoxymesterone [48]. Bleomycin cytotoxicity is increased by several membrane-acting drugs [55]. Illiger and Herdrich [56] have extensively reviewed many of the drug interactions encountered in cytotoxic chemotherapy.

The purpose of some experimental designs is to specifically test the interactions of drugs. For

example, there is considerable interest in the generation of drugs that may reverse the efflux activities of P-glycoprotein, the glycoprotein product of the MDR1 gene [57]. There is clear evidence that some reversing agents, if not all, alter the pharmacokinetics of cytotoxic drugs [58]. This may reflect modulation of normal P-glyco-protein function in the liver and other tissues, leading to an effective increase in drug exposure. Thus, increased activity of a cytotoxic agent requires careful evaluation.

One simple way to control for such effects is to compare equitoxic doses, *e.g.* the cytotoxic drug alone and in the presence of the reversing agent using schedules each of which produce an MTD. While this is a reasonable approach for pilot studies, directly assessing the pharmaco-kinetics of the cytotoxic drug with and without the reversing agent is definitive. Ultimately, such pharmacokinetic data may be required to clearly demonstrate that effects are not simply due to perturbations in the cytotoxic drug's mean serum concentration/plasma residence time.

Estimation of drug dosage and choice of starting dose/dose range

Where established drugs are to be used, well doc-umented protocols are available from the literature (Table 2). This is, at best, a general guide, since some rodent strains may be more or less sensitive to the toxic side effects of specific agents.

Identifying a dose regimen for unknown or experimental agents is frequently empirical. Often the initial studies are performed to obtain estimates of the MTD/LD_{10}. A simple dose es-calation study with a limited number of animals per group is a common strategy. The choice of starting dose to obtain an MTD estimate could be based on one of several criteria, such as the known *in vitro* toxicity, or toxicity of a closely related compound. Where agents under investi-gation are natural products or analogues thereof, *e.g.* phytochemicals, the levels of exposure in human populations may be available and enable

estimation of the starting dose. One simple approach, where resources are limited, is to do a pilot (dose range finding) study, using a broad range of doses but a small number of animals (one or two animals per dose). This has the advantage of limiting the number of animals that may be exposed to particularly toxic/lethal doses when the intent is to rapidly establish a toxic dose. A second, more definitive, follow-up study can then be done to obtain the MTD, using a limited number of mostly sublethal doses with larger numbers of animals per group. Animal usage can be further limited by restricting these follow-up studies only to those drugs that show antineoplastic activity and are identified for further evaluation in subsequent secondary screen-ing.

For preliminary toxicological analyses, *e.g.*, estimates of MTD, all animals should be mon-itored twice daily at a minimum. Recording animal body weights and food consumption twice weekly can identify the onset of some toxicities that are not immediately apparent. Loss of 10% of the starting body weight, or failure to gain body weight at the same rate as controls, provide useful endpoints for estimating an MTD. Many cytotoxic agents will produce more immediate effects, which are often apparent from the altered behavior of the animals, *e.g.* crouching, somno-lence, or reduced activity. These also can be used as endpoints to establish an MTD. Such effects may be transient, with the animals recovering within several hours if no further drug is admin-istered, or they can persist and lead to morbidity and death if prolonged over several days or weeks.

Where drug related deaths occur within several hours of administration, these data should not be used to establish the MTD, since toxicity could merely reflect sensitivity to the peak plasma concentration resulting from the bolus. Rather, the dosage and/or schedule should be modified, *e.g.* using lower doses and perhaps more frequent administration. This pattern of toxicity, *i.e.* schedule-independent with peak plasma level toxi-city, has been referred to as category III [59].

Other categories reflect whether toxicity is schedule dependent (category I) or independent (category II, where total dose determines toxicity) [60,61].

Hematologic toxicity is often assessed as a toxicological endpoint, but the transient suppression of some cell populations, and the timing of their likely recovery, need to be considered. It can be useful to measure white blood cell counts and hematocrits twice weekly, or as appropriate, on two or more individuals in each group. In many cases this can be done by retroorbital bleeding. However, the same animal should not be bled repetitively, since this can be sufficient to influence the parameters under investigation, independent of the treatment. It should also be noted that hematopoietic toxicity is not always evident from peripheral blood assays, and may require spleen colony formation analyses [49]. Additional evidence of toxicity may be apparent on determining other parameters, *e.g.* SPGT, total bilirubin, BUN, creatinine. Such detailed blood analyses are usually restricted to more intensive toxicological studies, where identifying the dose limiting or lethal toxicity is required. However, if the aim is to obtain an approximate MTD in a pilot study, it can be informative to sacrifice moribund or clearly affected animals by terminal bleeding under appropriate anesthesia. The blood can then be used for a wider panel of preliminary tests. It is usually routine to sacrifice all animals at the end of the study and perform necropsies with subsequent examination of the major tissues and organs for evidence of gross and/or micro-anatomic toxicity.

Site of inoculation, tumor-host interactions, and drug delivery

The site of tumor cell inoculation can significantly alter tumor growth and metastatic potential [62-65] and has previously been discussed in detail [28]. Cells re-inoculated into the site of the original tumor (orthotopic implantation) produce metastatic lesions more frequently than those inoculated elsewhere [64]. We routinely use the mammary fat pad area as the preferred inoculation site for mammary tumors [17]. Where orthotopic implantation is either inappropriate or not required, *s.c.* inoculation into the flank facilitates good responses for cytotoxic agents and *s.c.* inoculation into the back facilitates good responses for irradiation regimens [66].

The "tumor bed effect", which relates to modifications in the tissue at the site of implantation, has been most widely studied in relation to radiosensitivity but can also influence response to cytotoxic drugs [54]. Some tumor models utilize inoculation into preirradiated subcutaneous sites. Prior irradiation of normal tissues can significantly impair their ability to produce new vascular tissues in response to the tumor. Thus, drug delivery to the tumor can be reduced when tumors are grown in preirradiated sites. The "tumor bed effect" has been reviewed by Milas [54].

Techniques for the determination of *in vivo* antitumor activity

Several techniques have been used to determine the activity of antineoplastic agents against experimental tumors growing *in vivo*. These tend to fall into one of two main categories, *in situ* and excision assays. *In situ* assays are performed entirely *in vivo*. Tumors are inoculated into the appropriate host, treated *in vivo*, and the effects of drug treatment estimated either on various parameters of tumor growth, or on the duration of survival. Excision assays also are based on the treatment of tumors growing *in vivo*. However, the estimations of cytotoxicity require removal of the tumor for further evaluation. This can include estimating colony forming ability *in vitro*, or TD_{50} estimations of the number of treated cells required to form tumors on reinoculation into a second host. Each of these techniques has unique advantages and disadvantages. Irrespective of the tumor endpoint, many investigators include additional groups of tumor-bearing animals treated with a drug known to be active against the tumor model [67].

In situ assays — tumor growth delay

Perhaps the most widely used *in situ* technique determines the effect of drug treatment on the kinetics of tumor regrowth. Tumors are inoculated into the appropriate host, treatment being initiated when a specific tumor size is reached, and measured at regular intervals until both treated and untreated tumors have reached a predetermined size. Growth delay is assessed as the time difference between treated and control tumors to reach this predetermined size. This approach measures growth of tumors of the same size, an important consideration when Gompertzian kinetics are involved.

There are a number of considerations specific to the design of tumor growth delay assessments. The measurements should be performed on proliferating tumors, so that it is advisable to obtain some estimate of pretreatment tumor growth characteristics to enable tumor selection. For example, where the size at treatment is to be 6 mm, the range of tumor sizes for inclusion in combination chemotherapy studies might be ±1-2 mm in diameter. The issue of appropriate randomization of animals into each group is discussed elsewhere [68]. The optimal size at treatment will vary depending on the individual growth characteristics of the tumor cell line but should be sufficient to produce tumors that are easily measured yet not so large that they include significant areas of hypoxia or necrosis. The optimum endpoint size is close to the size at treatment, *e.g.* twice the treatment volume. This minimizes any effects of treatment on growth rate [69].

An alternative endpoint to growth delay for *in situ* techniques is overall survival. This method has proved reliable for the murine ascites tumors, since the growth properties and lethal tumor burden are well established. Survival can easily be compared with an untreated tumor-bearing population. Survival is a less reliable endpoint for many solid tumors. The majority of solid human breast tumor models exhibit a poor or unpredictable metastatic potential. Thus, tumor burden is almost exclusively provided by the primary tumor. In these cases, the lethal tumor burden (primary tumor), as a percent of total body weight, can be far in excess of that observed in humans. Some poorly vascularized tumors may increase in volume and yet contain a relatively stable volume of viable tissue.

Tumor measurement: area, volume, and weight

Tumor area and/or volume is usually recorded every 1-4 days, depending on the growth characteristics of the tumor. The length of the longest axis and the width perpendicular to the longest axis are sufficient to obtain tumor area. For easily accessible tumors, a third perpendicular measurement can be obtained to determine tumor volume. This is useful when it is apparent that palpable tumors are particularly irregular in shape and unlikely to meet the requirements for volume assessments from measurements of tumor area. For a more detailed description of tumor measurements see the accompanying article by Rygaard and Spang-Thomsen [70].

Tumor weight, as determined at necropsy, is used as an endpoint in some drug studies. However, it is generally inadvisable as the sole endpoint. While it provides an accurate measurement of the final tumor, it does not allow for assessments of the activity of the treatment on growth kinetics, being only a "snap shot" of the tumor's growth. For example, a cytotoxic and cytostatic treatment could produce the same reduction in final tumor weight. The cytotoxic treatment could have induced an initial complete remission (disappearance and regrowth of the tumor), implying a possible induction of cell death and the potential for an alternative dose/schedule to produce cure. The cytostatic agent could have decreased the rate of cell proliferation without inducing any significant cell kill. Where there is heterogeneity in initial cell volume, the ability to statistically demonstrate activity also may be compromised. This is less problematic when consecutive measurements are

obtained on each tumor, *e.g.* to obtain individual T_Ds.

It also is possible to estimate tumor weight/volume from caliper measurements of tumor area. Tumor volume can be obtained from area measurements by [71]:

$$\text{tumor weight (mg)} = \text{(length) (width}^2) / 2$$

where: measurements = mm for all equations.

Where the volume is estimated at necropsy and the area of necrosis can be measured, this is modified to [72]:

$$\text{tumor weight (mg)} = [(\text{length x width}^2) / 2] - [(\text{necrosis length x necrosis width}^2) / 2]$$

For nonspherical tumors, the volume can be estimated by [73]:

$$\text{volume} = 4/3\pi(\text{largest diameter}/2 \text{ x smallest diameter}/2)^{3/2}$$

Other approaches include measuring three perpendicular dimensions and simply multiplying the three estimates [74]. While obtaining these measurements is often feasible with *s.c.* tumors in the flanks or back of nude mice, there can be difficulty in obtaining reproducible measurements of height for some tumors. The underlying assumption that the tumors are "box shaped" appears reasonable, since assuming an ellipsoid shape does not appear to give a better estimate, at least for human lung tumors [75].

Where necessary, the ellipsoid volume can be estimated by [76]:

$$\text{volume} = 4/3\pi(\text{length}/2 \text{ x width}/2 \text{ x height}/2)$$

When such tumor volume/weight estimates are used, it is advisable to confirm the validity of the relationship by at least comparing the predicted tumor weights/volumes with the actual wet weights/volumes at necropsy at the end of the experiment.

Tumor volume measurements can be used to estimate T_D and growth delay. Data also can be transformed and plotted as changes in relative tumor volume. Relative tumor volume can be obtained from [77]:

$$\text{relative volume} = V_a/V_i$$

where V_a = volume at the start of treatment, and V_i = volume at day i.

Excision assays

The main purpose of excision assays is to directly estimate the fraction of cells in a tumor that have retained their clonogenicity (fractional cell survival). The greatest methodological variation in these assays relates to the technique chosen to determine clonogenicity. Probably the more widely utilized clonogenicity assays determine the number of tumor cells, in an *ex vivo* suspension, able to form anchorage-independent colonies *in vitro*. Clonogenicity is usually assessed in a semi-solid medium (agar or methyl cellulose). This approach has significant advantages over other excision assays in time, cost, and intra-experimental variability [55], and in reducing the numbers of animals.

The TD_{50} assay determines tumorigenicity *in vivo* and, therefore, the tumorigenic potential of the treated cell populations. The excised tumor is disaggregated and a dilution cloning technique used to determine the minimum number of cells required to form tumors upon reinoculation. While immunologic properties of the tumor can be problematic, reinoculation into an immune-compromised host can largely eliminate these problems [55]. Ascites, such as the MDA435/LCC6 [21], can be inoculated *i.p.* and treated *i.v.*, *s.c.*, or *p.o.* Surviving cells can easily be removed and reinoculated either into the mammary fat pads of recipient mice to assess tumorigenicity as solid tumors, or *i.p.* to assess tumorigenicity by ascites formation. This approach, while providing a rigorous determination of effects on *in vivo* clonogenicity, can require relatively large numbers of animals depending on the study design.

The need to appropriately reduce animal usage is likely to effectively eliminate many of these *in vivo/in vivo* experimental approaches. However, a modification of this approach is to

assess clonogenicity of *in vivo* treated tumors *in vitro*. Thus, cells are treated as solid tumors or ascites *in vivo*, with cells removed and fractional cell survival determined by an anchorage-independent colony formation assay *in vitro* (above). This has the disadvantage that *in vitro* clonogenicity is used as a surrogate for tumorigenicity. Some cells also may not immediately readapt to *in vitro* growth in a manner that allows for an adequate assessment of cell survival. However, for many cellular models there is no significant effect on estimates of cell survival, but there are advantages in substantially reduced costs and fewer animals required.

All excision assays suffer from the disadvantage that, at some point, cells are removed from the animal for manipulation. Thus, the critical tumor/host relationship is lost. The disaggregation of solid tumors, for either *in vivo* or *in vitro* analysis, can result in a cell population no longer representative of the tumor. The process of enzymatic digestion can reduce clonogenic potential and thereby appear to increase the activity of the treatment. The time of excision can also influence the results, since some cells may eventually repair potentially lethal damage [78]. One advantage of an ascites model for these types of studies is that the cells may not require damaging enzymatic and/or physical disaggregation.

Endpoint comparisons

The most important technical disadvantage of the excision compared with the *in situ* techniques is the necessity for removal and manipulation of the tumor prior to assessing cytotoxicity. The loss of the tumor microenvironment also is problematic, since the ability of normal cells to stimulate tumor regrowth may be a crucial factor in the apparent failure of some cytotoxic regimens. However, excision assays eliminate the problem of remaining dead (or reproductively dead) cells contributing to tumor volume, a concern that can arise with *in situ* assays. The tumor growth delay

assays have the disadvantage that the kinetics of tumor regrowth may be unpredictable for novel drug combinations. Tumors that regrow may have altered proportions of infiltrating normal cells that could cause an overestimation or underestimation of cell kill. The variability of intertumor regrowth patterns also can be problematic. Nevertheless, the *in situ* assays generally appear to be favored in the literature.

Death/survival as an endpoint

Many institutional animal care and use committees are restricting or eliminating death as an endpoint in drug screening and other *in vivo* studies. Nevertheless, some investigators may have experimental designs where death/morbidity/survival is a justified requirement of the study. Several related issues require consideration. Survival can be measured at a fixed time, *e.g.,* the proportion of animals remaining alive at a predetermined time point beyond when the last untreated animal dies, or the median duration of survival when all animals in all groups die within the observation time. When "cure" or the proportion of "long term survivors" are estimated, it is necessary to define the time point at which "cure" or "survival" is attributed. A major concern is the T_D, since sufficient time must be allowed for any significant number of remaining cells to proliferate to the point where a palpable tumor/ascites would be expected. A solid tumor with $T_D = 48$ hr may require up to four months of post treatment observation to establish "cure" [32]. Defining long term survivors (where death/morbidity is the primary endpoint) often is based on the duration of survival of mice bearing untreated tumors, *e.g.,* three times their mean or median survival. By this criterion, with a mean survival of 30 days in the untreated group, treated mice that survive for 90 days could be considered long term survivors [21]. When there are survivors, the data analysis procedures need to take this into account [68].

There is little ethical justification for using

death as an endpoint for solid tumors that are easily accessible for estimations of tumor growth delay or excision assays. In general, survival as an endpoint should probably be restricted to ascites models, and perhaps also to solid tumors known to achieve a lethal tumor burden within an appropriate and predictable time. Even for these tumors, it may be possible to substitute morbidity for death. We have found this to be a viable and more humane alternative in several ascites studies. While it requires knowledge of the time from the onset of morbidity to death, and evidence that this period is sufficiently consistent, this information can be obtained, in advance, on a relatively few animals.

Where possible, morbidity should be considered as a poetntial surrogate for death as an endpoint in survival analyses. To assist other investigators, the criteria used to define morbidity, and the verification of its applicability as a surrogate for death, should be reported.

Approaches to data analysis

Estimation of tumor doubling times

Estimates of T_D can be obtained from measurements of either tumor area or tumor volume. A detailed description of this approach can be found in the accompanying article by Rygaard & Spang-Thomsen [70]. The T_D for each individual tumor can be obtained, with those within an experimental group combined for further analysis, *e.g.,* use of an appropriate ANOVA to explore differences among different treatment groups. While there is evidence of investigators using simple linear regression models to estimate T_D from growth curves, this is potentially confounded by the Gompertzian nature of tumor growth kinetics [11,12,70]. However, in short term studies, particularly where the growth data approximate exponential growth, the Gompertzian model may reduce to an exponential growth model, which may provide a more efficient model for the data [68]. There also can be statistical concerns where

early deaths limit the data available for the adequate determination of a T_D [68].

Cell kill estimates from in situ *studies*

There are a number of methods for estimating cell kill from *in situ* analyses. For ascites models, estimations of percent increased life span (%ILS) provide an indication of therapeutic activity. %ILS can be estimated from:

$$\%ILS = 100(T\text{-}C)/C$$

where: T = median survival time of treated populations; C = median survival time of untreated control population of tumor bearing mice [67].

It should be noted that median survival times focus on the 50% survival estimates, and do not efficiently use all of the data in the survival curve. This can become problematic when there is substantial heterogeneity in the duration of survival in some treatment groups. One approach is to use the hazard ratio (HR) of death for the control group *versus* the treated group (J Hanfelt, personal communication). Thus, where there is considerable heterogeneity in survival data, %ILS may be better estimated from:

$$\%ILS = 100(HR\text{-}1)$$

where: HR = the hazard ratio of death for the control group *versus* the treated group. The HR can be obtained by Cox proportional-hazards regression analysis [79,80].

Estimates of cell kill can be derived from solid tumor growth curves, where repeated measurements are obtained over the period of time required for tumors to reach a predetermined size. Provided the T_D for untreated and regrowing (treated) tumors are equivalent, the cell kill can be estimated by a number of related formulae [81]. Cell kill can be determined from growth delay measurements using an estimate of the T_D [9] and the number of cell divisions required for each log increase in growth (3.32) [82].

Total cell kill can be estimated from [81]:

$$\log_{10} \text{cell kill} = (T\text{-}C)/(3.32)(T_D)$$

Cell kill/dose can be estimated from [81]:

$$\log_{10} \text{ cell kill/dose} = (T-C)/(3.32)(T_D)(n)$$

where: T = median time to predetermined size in treated populations; C = median time to predetermined size in untreated control populations; n = number of treatments.

Net cell kill can be estimated from [81]:

$$\text{Net } \log_{10} \text{ cell kill} =$$
$$(T-C)-(\text{duration of treatment})/(3.32)(T_D)$$

The specific growth delay for solid tumors can be estimated from the times taken for both treated (T) and control (C) tumors to reach a predetermined size [66]. If the specific growth delay value exceeds 2, the tumor model is often considered responsive. This is generally considered the most important estimate of antitumor activity, and can be reported along with the total cell kill estimated from the same primary data (above).

Specific growth delay can be estimated from [77,83]:

$$\text{Specific growth delay} = (T-C)/C$$

Where the T_D of tumor regrowth is significantly different from the T_D of untreated tumors, these estimates are invalid. For example, the T_D of treated populations may be slower than equivalent untreated tumors following irradiation [69]. Other limitations of these analyses are indicated when treatment is begun shortly after tumor cell inoculation, where a significant immune component is suspected, or where the treatments are close to being curative [72]. The degree of response in tumors that exhibit alterations in the T_D following treatment can be more appropriately expressed as delay/doubling time [69].

Determination of synergy

Studies are often performed to determine the nature of the interaction between two or more treatments. Many investigators claim synergistic interactions without having performed the necessary analyses. Several authors have more clearly defined these terms and described the conditions required to determine whether an interaction is synergistic, additive, or antagonistic [84-86].

Synergism is a mathematically defined interaction, most widely determined from isobologram analyses. Construction of classical isobolograms (isoeffect curves) for the determination of additivity, synergy, and antagonism generally requires an initial estimation of the dose response curve of each agent alone and dose responses of one agent in the presence of each of several concentrations of the second agent [72]. While this may be feasible for routine *in vitro* studies [84], classical isobologram analyses generally require too much information to be readily applicable to *in vivo* data. A minimum requirement for *in vivo* studies has been suggested, using five doses of each drug alone and three or four combination treatments using intermediate doses [87]. An interaction index (I_x) can then be estimated from the isobologram equation [88,89]:

$$I_x = (d/D) + (t/T)$$

where ED_n = some fixed level of activity, D and T = dose of each drug alone required to produce ED_n, and d and t = dose for each drug in combination that produce ED_n. I_x values of <1 indicate synergy, a value of 1 reflects additivity, while values >1 indicate antagonism. Ideally, each ED_n value should be estimated by performing appropriate probit or logit analyses of the dose reponse data [90,91].

An alternative experimental design is to fix the dose of the first drug and vary the concentration of the second [92]. This approach has not been widely applied to *in vivo* studies, and its ability to adequately define the nature of drug interactions does not appear to have been extensively confirmed. Nevertheless, it could be applied to *in vivo* studies and may be worthy of consideration.

Application of the median effect analysis is one of several alternative approaches to the classical isobologram approach for assessing synergy [85]. While this method may be amenable for use in *in vivo* studies, this has yet to be firmly

established. Other related approaches to assessing synergy also are reported [93] but their application to *in vivo* studies also remains to be confirmed. The concern is not the validity of the approaches, which is beyond the scope of the current article, but how appropriate *in vivo* experiments could be designed, within the constraints of time, cost, and appropriate animal usage, to produce sufficient data to generate statistically reliable estimates of the nature of the drug interactions.

Where one drug has no activity, synergy is more readily determined [93] and may simply require statistical evidence that the combination is significantly different from the inhibitory drug alone. This simplistic approach is potentially confounded when the "inactive" compound is active at higher concentrations. If the dose used was at a theoretical ED_{10}, *i.e.*, activity was present but could not be detected because of the sensitivity of the assay, assumptions of synergy could well be invalid. Thus, if this approach is to be used, it should be at least restricted to doses well below the minimally active dose, or better yet, restricted to studies where one compound is known to have no antineoplastic activity.

Where resources are available, isobologram-based approaches are preferred. However, some interactive definitions may be approximated from limited data as follows [94,95]:

Antagonistic	$(AB)/C > (A/C) \times (B/C)$
Additive	$(AB)/C = (A/C) \times (B/C)$
Synergistic	$(AB)/C < (A/C) \times (B/C)$

where A = response to treatment 1; B = response to treatment 2; C = response to no treatment/vehicle; AB = combination of treatments A and B. Some investigators define a fourth interaction category (subadditive = $(AB)/C > [(A/C) \times (B/C)]$ < $[B/C]$ (where $B/C > A/C$). However, a subadditive interaction is essentially antagonistic, making the utility of this term somewhat unclear from a pharmacologic perspective.

The application of this approach may vary depending on the endpoint and experimental de-sign chosen for a particular study. For excision assays using *in vitro* colony formation as an endpoint, the response to treatment would be represented by the colony forming ability or surviving fraction. Growth delay assays could utilize the estimates of cell kill/survival described above or T_D parameters. A description of approaches to the statistical analysis of data obtained from these studies can be found in the accompanying article by Hanfelt [68].

These terms are approximations, at best, when compared with more classical isobologram approaches, and should be used with some caution. The outcome provides only a general approximation of the nature of the interaction, and should not be considered definitive. While reasonable approximations may be obtained where tumors approximate logarithmic growth, the analysis does not take into account the shape of the respective dose response curves, a central component of isobologram approaches for determining the nature of drug interactions. This could lead to both underestimates and overestimates of potential synergy or antagonism.

Therapeutic synergism has been defined as occurring when the response of a combination is in excess of the maximum response of either drug alone (*e.g.* A = 100%, B = 150%, A+B = 200%) at equitoxic doses [67,96,97]. The term "clinical synergism" also has been applied when a combination chemotherapy regimen is curative [96]. These are not particularly informative definitions from an interpretive or mechanistic perspective, and could be applicable when the "true" biochemical interaction is only additive or even antagonistic.

In practice, a drug combination can be curative and yet produce a nonsynergistic interaction. If two drugs with different mechanisms of action each kill 10^6 cells, a tumor of 10^9 cells could still be cured by an additive or even antagonistic interaction [96]. Thus, the determination of synergy or antagonism is not required where the purpose is simply to show that a combination of two or more drugs is curative relative to either agent administered alone.

The toxicity of a particular combination should be considered when determining the nature of an interaction between two or more drugs. In order to make appropriate comparisons, the treatments should be approximately equitoxic [97]. For example, the improved antitumor activity of a combination regimen which is accompanied by significantly increased toxicity might be no more effective than a higher dose of either agent alone that produced equivalent toxicity.

Some suggestions for statistical approaches to data analysis and the assessment of activity

The statistical analytical procedures used to explore data from animal studies is dependent on the study design. Statistical approaches will be dealt with only cursorily, since a detailed discussion of methods and approaches is provided elsewhere in this issue [68]. Readers are encouraged to consult this and other articles which deal with endpoints and considerations in depth beyond the scope of this section. While the use of some specific tests is suggested below, these should be used with some caution, since the primary data may violate assumptions implicit within the analyses. For example, some of these tests described below require that the data approximate a normal or other distributional form. When this is not apparent, or cannot be achieved by transformation, e.g. converting to $\log_{10}$, nonparametric analyses may be required. For investigators unfamiliar with statistical analyses, there is no substitution for consultation with a biostatistician.

One of the key first steps in the design of animal experiments should be the determination of appropriate group sizes. The number of animals per group will depend on the endpoint and the magnitude of the expected response. In this regard, it is necessary to perform appropriate power estimates to ensure that the design will have sufficient statistical power to adequately identify significant or nonsignificant differences. This is discussed in some detail in the accompanying article by Hanfelt [68]. It is important to ensure that group size is sufficient to maintain power when some animals may either die from other causes, e.g. drug toxicity or secondary infection, or bear tumors that do not exhibit appropriate pretreatment kinetics. For pilot/exploratory studies, where more definitive follow-up studies are planned, this is of lesser concern. The data from such pilot studies can often provide useful information on the expected response rate and toxicity, thereby facilitating the generation of potentially more relevant power estimates.

Tumor growth delay is generally assessed in terms of the time necessary for tumor growth in each group to increase from the initial size at the time of treatment to a larger, predetermined size [66,81,98]. The most common approaches are to compare the T/C or T-C values. Both parameters are usually based on median values, with zeros included for the T/C estimates and "tumor-free cures/tumor free survivors" being excluded from the estimates and analyzed separately. A T/C value $\leq 42\%$ is generally required to demonstrate activity [67]. The necessity to exclude cures may be problematic if it affects the power of the analysis. As discussed by Hanfelt [68], the use of median times also may be inefficient, with longitudinal analyses providing a more effective use of the data.

There are several other ways to explore data from tumor growth delay studies. For example, survival analyses also can be used to assess "time-to-event" endpoints, e.g. time to reach a predetermined size. A further approach is to use a repeated measures ANOVA to compare tumor size at each time point across the analysis. This may be most applicable if a significant number of tumors in a treatment group do not reach the predetermined size in the control group. Alternatively, tumor doubling times can be estimated for each individual tumor in each group by applying Gompertzian kinetic analyses, such as those performed by the GROWTH software [70]. Tumor doubling times can be compared among experimental groups by either ANOVA or multivariate ANOVA [99]. This approach also is useful for

cytostatic treatments, *e.g.,* hormones or anti-hormones, and to ensure that the kinetics of regrowth are appropriate for cell kill estimates.

For tumor excision assays, *in vitro* colony forming ability among groups, or *in vivo* tumor incidence if reinoculated into recipient mice, can be compared by either ANOVA or multivariate ANOVA.

Percent increased life span (%ILS) is the most widely used activity measure for ascites tumors. Since some animals may survive, survival curves may be estimated using the Kaplan-Meier approach [100], and differences in survival between treatment groups estimated by the Log-Rank test [101]. Where early deaths occur from drug toxicity and later deaths from tumor burden, a model more sensitive to early events may be necessary [68]. In general, a %ILS ≥27% in the P388 ascites model is considered the minimum for activity when both drug and tumor are administered *i.p.* When drugs are administered *i.v.* to an *i.p.* tumor, a %ILS >40% is considered sufficient to demonstrate activity [67].

Tumor incidence, proportion of survivors, or long term survivors, *e.g.* 2x2 analysis using single treatment and control, can be compared among groups by χ^2. If more than one site is used per mouse, General Estimating Equation methods are required to account for any lack of independence of tumors within animals [68]. If the number of observations is small, a Fishers exact test or Pearson's χ^2 test may be used.

Body or tumor weights, where the data are continuous and randomly distributed, can be compared by ANOVA followed by a multiple comparison *post hoc* test such as Duncan's multiple range test [102]. Where it is required to merely compare several individual groups with the same control, *e.g.* dose-response analysis, and where the group sizes are equal, Dunnet's t-test can be used [103]. Where group sizes are different, Scheffe's multiple comparison test can be applied [104]. However, it should be noted that some endpoints also are associated with several variables. For example, organ weights increase with increasing body weight. For such endpoints,

analysis of covariance approaches are required [105].

Conclusions

As indicated in the introduction, many of the issues are raised to assist investigators in approaching some of the major concerns that arise in designing studies to test the activity of cytotoxic agents. There are alternative approaches to several of these issues, but a detailed discussion is beyond the scope of the current article. Some are described in detail elsewhere in this journal issue, and others can be found in many of the publications cited herein. While the relative importance of many of the topics addressed will vary with the hypothesis and endpoint chosen, some issues will apply irrespective of these, *e.g.* the need to design experiments with sufficient statistical power.

Acknowledgments

References

1. Goldie JH, Coldman AJ: A mathematical model for relating the drug sensitivity of tumors to the spontaneous mutation rate. Cancer Treat Rep 63: 1727-1733, 1979

2. Beatson GT: On the treatment of inoperable cases of carcinoma of the mamma: suggestions from a new method of treatment, with illustrative cases. Lancet ii:104-107, 1896

3. Cole MP, Jones CTA, Todd IDH: A new antioestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 25:270-275, 1971

4. Howell A, DeFriend DJ, Robertson JFR, Blamey

RW, Anderson L, Anderson E, Sutcliffe FA, Walton P: Pharmacokinetics, pharmacological and anti-tumor effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. Br J Cancer 74:300-308, 1996

5. Bonadonna G: Does chemotherapy fulfill its expectations in cancer treatment? Ann Oncol 1:11-21, 1990

6. Boyd MR: Status of the NCI preclinical antitumor drug discovery screen. PPO Updates 3 (10):1-12, 1989

7. Richardson VJ, Ford CHJ, Tsaltas G, Gallant ME: Doxorubicin-anti-carcinoembryonic antigen immuno-conjugate activity in vitro. Eur J Cancer Clin Oncol 25:633-640, 1989

8. Griffin JD: Hemopoietins in oncology: factoring out myelosuppression. J Clin Oncol 7:151-155, 1989

9. Skipper HE, Schabel FM, Wilcox WS: Experimental evaluation of potential anticancer agents. XII. On the criteria and kinetics associated with "curability" of experimental leukemia. Cancer Chemother Rep 35:1-111, 1964

10. Wilcox WS: The last surviving cancer cell: the chances of killing it. Cancer Chemother Rep 50:541-542, 1966

11. Norton L, Simon R: Tumour size, sensitivity to therapy, and design of treatment schedules. Cancer Treat Rep 61:1307-1317, 1977

12. Green JA: After Goldie-Coldman — where now? Eur J Cancer Clin Oncol 25:913-916, 1989

13. Schabel FM: Concepts from systemic treatment of micrometastases. Cancer 35:15-24, 1975

14. De Vita VT: The relationship between tumor mass and response to chemotherapy. Implications for surgical adjuvant treatment of cancer. Cancer 51: 1209-1220, 1983

15. Tannock IF: Principles of cell proliferation: cell kinetics. *In:* De Vita VT, Hellman S, Rosenberg SA (eds) Cancer: Principles and Practice of Oncology. J.B. Lippincott, Philadelphia, 1989, pp 3-13

16. Steel GG: Cell loss as a factor in the growth rate of human tumours. Eur J Cancer 3:381 1967

17. Clarke R, Brünner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB: Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. Proc Natl Acad Sci USA 86:3649-3653, 1989

18. Brünner N, Boulay V, Fojo A, Freter C, Lippman ME, Clarke R: Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283-290, 1993

19. Brünner N, Boysen B, Jirus S, Skaar TC, Holst-

Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SAW, Clarke R: MCF7/LCC9: an anti-estrogen resistant MCF-7 variant where acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early crossresistance to the non-steroidal antiestrogen tamoxifen. Cancer Res 57:3486-3483, 1997

20. Harris JR, Hellman S: Natural history of breast cancer. *In:* Harris J, Lippman ME, Morrow M, Hellman S (eds) Diseases of the Breast. Lippincott-Raven, Philadelphia, 1996, pp 375-391

21. Leonessa F, Green D, Licht T, Wright A, Wingate-Legette K, Lippman J, Gottesman MM, Clarke R: MDA435/LCC6 and MDA435/LCC6^{MDR1}: ascites models of human breast cancer. Br J Cancer 73:154-161, 1996

22. De Vita VT: Principles of chemotherapy. *In:* De Vita VT, Hellman S, Rosenberg SA (eds) Cancer Principles and Practice of Oncology. J.B. Lippincott, Philadelphia, 1989, pp 276-300

23. Winograd B: New drug development. *In:* Boven E, Winograd B (eds) The Nude Mouse in Oncology Research. CRC Press, Boca Raton, 1991, pp 305-316

24. Inaba M, Kobayashi T, Tashiro T, Sakurai Y: Pharmacokinetic approach to rational therapeutic doses for human tumor-bearing nude mice. Gann 79:509-515, 1988

25. Kubota T, Inoue S, Furukawa T, Ishibiki K, Kitajima M, Kawamura E, Hoffman RM: Similarity of serum-tumor pharmacokinetics of antitumor agents in man and nude mice. Anticancer Res 13:1481-1484, 1993

26. Braakhuis BJM, van Dongen GAMS: Limitations for treatment studies. *In:* Boven E, Winograd B (eds) The Nude Mouse in Oncology Research. CRC Press, Boca Raton, 1991, pp 291-303

27. Dodwell DJ, Gurney H, Thatcher N: Dose intensity in cancer chemotherapy. Br J Cancer 61:789-794, 1990

28. Clarke R: Human breast cancer cell line xenografts as models of breast cancer: the immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast Cancer Res Treat 39: 69-86, 1996

29. Clarke R, van den Berg HW, Kennedy DG, Murphy RF: Reduction of the antimetabolic and antiproliferative effects of methotrexate by 17β-estradiol in a human breast carcimoma cell line (MDA-MB-436). Eur J Cancer Clin Oncol 19:19-24, 1983

30. Clarke R, van den Berg HW, Kennedy DG, Murphy RF: Oestrogen receptor status and the response of human breast cancer cells to a combination of methotrexate and 17β-estradiol. Br J Cancer 51:365-369, 1985

31. Antoniades K, Spector H: Quantitative estrogen

receptor values and growth of carcinoma of the breast before surgical intervention. Cancer 50:793-796, 1982

32. Gabrilove JL, Jakubowski A, Scher H, Sternberg C, Wong G, Grous J, Yagoda A, Fain K, Moore MA, Clarkson B, et al: Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N Engl J Med 318:1414-1422, 1988

33. Goldman CA, Skinnider LF, Maksymiuk AW: Interferon instillation for malignant pleural effusions. Ann Oncol 4:141-145, 1993

34. Henderson IC, Shapiro CL: Adjuvant chemotherapy: an overview. *In:* Powles T, Smith IE (eds) Medical Management of Breast Cancer. Dunitz, London, 1991, pp 197-215

35. Henderson IC: Chemotherapy for metastatic disease. *In:* Harris JR, Hellman S, Henderson IC, Kinne DW (eds) Breast Diseases. J.B.Lippincott Co., Philadelphia, 1991, pp 604-665

36. Garewal HS: Mitomycin C in the chemotherapy of advanced breast cancer. Semin Oncol 15 suppl 4:74-79, 1988

37. Donehower RC, Rowinsky EK: Paclitaxel. PPO Updates 8 (10):1-16, 1994

38. Trock BJ, Leonessa F, Clarke R: Multidrug resistance in breast cancer: a meta analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst 89:917-931, 1997

39. Vickers PJ, Dickson RB, Shoemaker R, Cowan KH: A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone independent tumor growth. Mol Endocrinol 2:886-892, 1988

40. Zyad A, Bernard J, Clarke R, Tursz T, Brockhaus M, Chouaib S: Human breast cancer cross-resistance to TNF and adriamycin: relationship to MDR1, MnSOD and TNF gene expression. Cancer Res 54:825-831, 1994

41. Doroshow JH, Akman S, Esworthy S, Chu FF, Burke T: Doxorubicin resistance is conferred by selective enhancement of intracellular glutathione peroxidase or superoxide dismutase content in human MCF-7 breast cancer cells. Free Radic Res Commun 12-13 Pt 2:779-781, 1991

42. Iwamoto S, Takeda K: Possible cytotoxic mechanisms of TNF in vitro. Hum Cell 3:107-112, 1990

43. Batist G, Tuple A, Sinha BK, Katki AG, Myers CE, Cowan KH: Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. J Biol Chem 261:15544-15549, 1986

44. Sinha BK, Mimnaugh EG, Rajagopalan S, Myers CE:

Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. Cancer Res 49:3844-3848, 1989

45. Corbett TH, Valeriote FA, Baker LH: Is the P388 murine tumor no longer adequate as a drug discovery model? Invest New Drugs 5:3-20, 1987

46. Clarke R, Currier S, Kaplan O, Lovelace E, Boulay V, Gottesman MM, Dickson RB: Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells. J Natl Cancer Inst 84:1506-1512, 1992

47. Nooter K, de la Riviere BG, Look NP, van Wingerden KE, Henzen-Logmans SC, Scheper RJ, Flens MJ, Klijn JGM, Stoter G, Foekens JA: The prognostic significance of excpression of the multidrug resistance-associated protein (MRP) in primary breast cancer. Br J Cancer 76:486-493, 1997

48. Clarke R, Brünner N, Thompson EW, Glanz P, Katz D, Dickson RB, Lippman ME: The inter-relationships between ovarian-independent growth, anti-estrogen resistance and invasiveness in the malignant progression of human breast cancer. J Endocrinol 122:331-340, 1989

49. Double JA, Bibby MC: Therapeutic index: a vital component in selection of anticancer agents for clinical trial. J Natl Cancer Inst 81:988-994, 1989

50. Rosenhoff SH, Bull JM, Young RC: The effect of chemotherapy on the kinetics and proliferative capacity of normal and tumorous tissues in vivo. Blood 45:107-118, 1975

51. Myers CE, Young RC, Chabner BA: Kinetic alterations by 5-fluorouracil in bone marrow, intestinal mucosa, and tumor. Cancer Res 36:1653-1658, 1976

52. Kennedy DG, Clarke R, van den Berg HW, Murphy RF: The kinetics of methotrexate polyglutamate formation and efflux in a human breast cancer cell line (MDA-MB-436): the effect of insulin. Biochem Pharmacol 32:41-46, 1983

53. Shepherd R, Harrap KR: Modulation of the toxicity and antitumour activity of alkylating drugs by steroids. Br J Cancer 45:413-420, 1982

54. Martin AD, Beer RW, Bosanquet AG, Gilby ED: The effect of alkylating agents and other drugs on the accumulation of melphalan by murine L1210 leukaemia cells in vitro. Biochem Pharmacol 31:2727-2732, 1982

55. Mizuno S, Ishida A: Potentiation of bleomycin cytotoxicity by membrane-interacting drugs and increased calcium ions. Biochem Biophys Res Commun 107:1021-1027, 1982

56. Illiger HJ, Herdrich K: Drug interactions in the therapy of malignant diseases. Springer-Verlag, Munich, 1987

57. Ueda K, Clark DP, Chen CJ, Roninson IB, Gottesman MM, Pastan I: The human multidrug resistance (MDR1) gene. cDNA cloning and transcription initiation. J Biol Chem 262:505-508, 1987

58. Horton JK, Thimmaiah KN, Houghton JA, Horowitz ME, Houghton PJ: Modulation by verapamil of vincristine pharmacokinetics and toxicity in mice bearing human tumor xenografts. Biochem Pharmacol 38:1727-1736, 1989

59. Pazdur R, Redman BG, Corbett TH, Phillips M, Baker LH: Phase I trial of spiromustine (NSC 172112) and evaluation of toxicity and schedule in a murine model. Cancer Res 47:4213-4217, 1987

60. LoRusso P, Wozniak AJ, Polin L, Capps D, Leopold WR, Werbel LM, Biernat L, Dan ME, Corbett TH: Antitumor efficacy of PD115934 (NSC 366140) against solid tumors of mice. Cancer Res 50:4900-4905, 1990

61. Corbett RJ, Leopold WR, Dykes DJ, Roberts BJ, Griswold DP, Schabel FM: Toxicity and anticancer activity of a new triazine antifolate (NSC 127755). Cancer Res 42:1707-1715, 1982

62. Meyvisch C: Influence of implantation site on formation of metastases. Cancer Metastasis Rev 2:295-306, 1983

63. Volpe JPG, Milas L: Influence of tumor transplantation methods on tumor growth rate amd metastatic potential of solitary tumors derived from metastases. Clin Exp Metastasis 8:381-389, 1990

64. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ: Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res 48:6863-6871, 1988

65. Kozlowski JM, Fidler IJ, Campbell D, Xu Z-L, Kaighn ME, Hart IR: Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res 44:3522-3529, 1984

66. Yamamoto S, Tanaka H, Kanamori T, Nobuhara M, Namba M: In vitro studies on potentiation of cytotoxic effects of anticancer drugs by interferon on a human neoplastic cell line (HeLa). Cancer Lett 20:131-138, 1983

67. Bissery M-C, Vrignaud P, Lavelle F: Preclinical profile of doxetael (Taxotere): efficacy as a single agent in combination. Semin Oncol 22:3-16, 1995

68. Hanfelt JJ: Statistical approachs to experimenal design and data analysis of in vivo studies. Breast Cancer Res Treat 46:279-302, 1997

69. Begg AC: Principles and practices of the tumor growth delay assay. In: Kallman RF (ed) Rodent Tumor Models in Experimental Cancer Therapy. Pergamon Press, New York, 1987, pp 114-121

70. Rygaard K, Spang-Thompsen M: Quantitation and gompertzian analysis of tumor growth. Breast Cancer Res Treat 46:303-312, 1997

71. Osieka R, Houchens DP, Goldin A, Johnson RK: Chemotherapy of human colon cancer xenografts in athymic nude mice. Cancer 40:2640-2650, 1977

72. Manda T, Nishigaki F, Mukumoto S, Masuda K, Nakamura T, Shimomura K: The efficacy of combined treatment with recombinant human tumor necrosis factor-α and 5-fluorouracil is dependent on the development of capillaries in tumor. Eur J Cancer Clin Oncol 26:93-99, 1991

73. Villa E, Dugani A, Fantoni E, Camellini L, Buttafoco P, Grottola A, Pompei G, De Santis M, Ferrari A, Manenti F: Type of estrogen receptor determines response to antiestrogen therapy. Cancer Res 56:3883-3885, 1996

74. Jefferson MF, Pendelton N, Faragher EB, Dixon GR, Myskow MW, Horan MA: 'Tumor volume' as a predictor of survival after resection of non-small-cell lung cancer (NSCLC). Br J Cancer 74:456-459, 1996

75. Clelland CA: Tumour volume. Br J Cancer 75:464-465, 1997

76. Binks S, Clelland CA, Layton C: A comparison of pathological methods of measuring lung cancer volume. J Clin Pathol 49:654-656, 1996

77. Silbermann MH, van den Vecht B, Stoter G, Nooter K, Verweij J: Combination therapy of ACNU and ifosfamide in tumor bearing mice with M2661 breast cancer, B16 melanoma or C38 colon cancer. Eur J Cancer 26:321-325, 1990

78. Namba M, Yamamoto S, Tanaka H, Kanamori T, Nobuhara M, Kimoto T: In vitro and in vivo studies on potentiation of cytotoxic effects of anticancer drugs or cobalt 60 gamma ray by interferon on human neoplastic cells. Cancer 54:2262-2267, 1984

79. Cox DR: Regression models and life tables (with discussion). J Royal Stat Soc B 34:187-220, 1972

80. Armitage P, Berry G: Statistical Methods in Medical Research. Blackwell Scientific Publications, Oxford, 1994, pp 484-489

81. Corbett TH, Valeriote FA: Rodent models in experimental chemotherapy. In: Kallman RF (ed) Rodent Tumor Models in Experimental Cancer Therapy. Pergamon Press, New York, 1987, pp 233-247

82. Hayflick L: Subculturing human diploid fibroblasts. In: Kruze PF, Patterson MK (eds) Tissue Culture: Methods and Applications. Academic Press, New York, 1973, pp 220-223

83. Steel GG, Courtney VD, Peckham MJ: The response of a variety of human tumour xenografts. Br J Cancer 47:1-13, 1983

84. Berenbaum MC: What is synergy? Pharmacol Rev 41:93-141, 1989

85. Rideout DC, Chou T-C: Synergism, antagonism, and potentiation in chemotherapy: an overview. *In:* Chou T-C, Rideout DC (eds) Synergism and Antagonism in Chemotherapy. Academic Press, San Diego, 1991, pp 3-53

86. Wampler GL, Carter WH, Campbell ED, Keefe PA: Relationships between various uses of antineoplastic drug-interaction terms. Cancer Chemother Pharmacol 31:111-117, 1992

87. Machado SG, Robinson GA: A direct, general approach based on isobolograms for assessing the joint action of drugs in pre-clinical experiments. Stats Med 13:2289-2309, 1994

88. Berenbaum MC: Synergy, additivism and antagonism in immunosuppression. A critical review. Clin Exp Immunol 28:1-18, 1977

89. Leonessa F, Jacobson M, Boyle B, Lippman J, McGarvey M, Clarke R: The effect of tamoxifen on the multidrug resistant phenotype in human breast cancer cells: isobologram, drug accumulation and gp-170 binding studies. Cancer Res 54:441-447, 1994

90. Govindarajulu Z: Statistical Techniques in Bioassay. Karger, Basel, 1988, pp 1-162

91. Armitage P, Berry G: Statistical Methods in Medical Research. Blackwell Scientific Publications, Oxford, 1994, pp 392-424

92. Deen DF, Williams ME: Isobologram analysis of X-ray-BCNU interactions *in vitro*. Radiat Res 79:483-491, 1979

93. Greco WR, Park HS, Rustum YM: Application of a new approach for the quantitation of drug synergism to the combination of cis-diamminedichloroplatinum and 1-β-D-arabinofuranosylcytosine. Cancer Res 50: 5318-5327, 1990

94. Aapro MS, Alberts DS, Salmon SE: Interactions of human leukocyte interferon with *Vinca* alkaloids and other chemotherapeutic agents against human tumors in clonogenic assay. Cancer Chemother Pharmacol 10:161-166, 1983

95. Marth C, Daxenbichler G, Dapunt O: Synergistic antiproliferative effect of human recombinant interferons and retinoic acid in cultured breast cancer cells. J Natl Cancer Inst 77:1197-1202, 1986

96. Valeriote F, Lin H-S: Synergistic interactions of anticancer agents: a cellular perspective. Cancer Chemother Rep 59:895-901, 1975

97. Corbett TH, Griswold DP, Wolpert MK, Vendetti JM, Schabel FM: Design and evaluation of combination chemotherapy trials in experimental animal tumor systems. Cancer Treat Rep 63:799-801, 1979

98. Moulder JE, Rockwell S: Comparison of tumor assay methods. *In:* Kallman RF (eds) Rodent Tumor Models in Experimental Cancer Therapy. Pergamon Press, New York, 1987, pp 272-278

99. Heitjan DF, Manni A, Santen RJ: Statistical analysis of in vivo tumor growth experiments. Cancer Res 53:6042-6050, 1993

100. Kaplan EL, Meier P: Non-parametric estimations from incomplete observations. J Am Stat Assoc 53: 457-481, 1958

101. Mantell N: Evaluation of survival data and two new rank order stastistics arising in its consideration. Cancer Chemother Rep 50:163-170, 1966

102. Duncan DB: Multiple range and multiple F tests. Biometrics 11:1-42, 1955

103. Dunnett CW: New tables for multiple comparisons with a control. Biometrics 20:482-492, 1964

104. Scheffe H: The analysis of variance. Wiley, New York, 1959

105. Gad S, Weil CS: Statistics and Experimental Design for Toxicologists. Telford Press, Caldwell NJ, 1988

106. Thompson EW, Paik S, Brünner N, Sommers C, Zugmaier G, Clarke R, Shima TB, Torri J, Donahue S, Lippman ME, Martin GR, Dickson RB: Association of increased basement membrane-invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 150:534-544, 1992

107. Soule HD, Vasquez J, Long A, Albert S, Brennan M: A human cell line from a pleural effusion derived from a human breast carcinoma. J Natl Cancer Inst 51:1409-1416, 1973

108. Cailleau R, Young R, Olive M, Reeves WJ: Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53:661-674, 1974

109. Price JE, Polyzos A, Zhang RD, Daniels LM: Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 50:717-721, 1990

110. Cailleau R, Olive M, Cruciger QVA: Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. In Vitro 14:911-915, 1978

111. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ: Establishment and characterization of a cell line of human carcinoma origin. Eur J Cancer 15:659-670, 1979

112. Clarke R, Leonessa F, Brunner N, Thompson EW: *In vitro* models of human breast cancer. *In:* Harris JR, Hellman S, Lippman ME, Morrow M (eds) Diseases of the Breast. J.B. Lippincott, Philadelphia, 1996, pp 245-261

113. Macubbin DL, Wing KR, Mace KF, Ho RLX, Ehrke MJ, Mihich E: Adriamycin-induced modulation of host defenses in tumor-bearing mice. Cancer Res 52:3572-3576, 1992

114. Gorelik E, Ovejera A, Shoemaker R, Jarvis A, Alley M, Duff R, Mayo J, Herberman R, Boyd M: Micro-

encapsulated tumor assay: new short term assay for *in vivo* evaluation of the effects of anticancer drugs on human tumor cell lines. Cancer Res 47:5739-5747, 1987

115. Chandrasekaran B, Capizzi RL, Kute TE, Morgan T, Dimling J: Modulation of the metabolism and pharmacokinetics of 1-β-D-arabinofuranosylcytosine by 1-β-D-arabinofuranosyluracil in leukemic mice. Cancer Res 49:3259-3266, 1989

116. Jiminez JJ, Yunis AA: Protection from 1-β-D-arabinofuranosylcytosine-induced alopecia by epidermal growth factor and fibroblast growth factor in the rat model. Cancer Res 52:413-415, 1992

117. Nielsen LL, Gurnani M, Tyler RD: Evaluation of the *wap-ras* transgenic mouse as a model system for testing anticancer drugs. Cancer Res 52:3733-3738, 1992

118. Spang-Thomsen M, Brünner N, Engelholm SA, Vindelov L: Estimation by flow cytometric DNA analysis of the effect of radiotherapy, hormone therapy and chemotherapy on human tumors grown in nude mice. In: Wu B, Zheng J (eds) Immune-Deficient Animals: 4th Workshop on Immune-Deficient Animals in Experimental Research. Karger, Basel, 1984, pp 409-415

119. Berger DP, Winterhalter BR, Fiebig HH: Conventional chemotherapy. *In:* Boven E, Winograd B (eds) The Nude Mouse in Onclogy Research. CRC Press, Boca Raton, 1991, pp 165-184

120. Miller BE, Machemer T, Lehotan M, Heppner GH: Tumor subpopulation interactions affecting melphalan sensitivity in palpable mouse mammary tumors. Cancer Res 51:4378-4387, 1991

121. Kerpel-Fronius S, Verwey J, Stuurman M, Kanyar B, Lelieveld P, Pinedo HM: Pharmacokinetics and toxicity of mitomycin C in rodents, given alone, in combination, or after induction of microsomal metabolism. Cancer Chemother Pharmacol 22:104-108, 1988

122. Imai R, Morimoto M, Marumo H, Kobayashi T, Tsuoro T, Inaba M, Tsukagoshi S, Sakurai Y: Antitumor activity of 7-N-(*p*-hydroxyphenyl)-mitomycin C in experimental tumor systems. Gann 72: 944-949, 1981

123. Los G, Mutsaers PHA, van der Vijgh WJF, Baldew GS, De Graaf PW, McVie JG: Direct diffusion of *cis*-diamminedichloroplatinum(II) in intraperitoneal rat tumors after intraperitoneal chemotherapy: a comparison with systemic chemotherapy. Cancer Res 49:3380-3384, 1989