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Sensitivity of Normal Human Bone Marrow Myeloid Progenitor Cells to Anthracycline, Cisplatin, Anthracene and Flavone Acetic Acid Derivatives, and its Relevance for the Prediction of Human Plasma Concentrations of Anticancer Drugs*

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Abstract—*Many* in vitro *investigations with anticancer agents are performed at concentrations equal to the peak concentrations or fractions of the peak concentrations achieved in human plasma after administration of these agents. In an effort to develop an* in vitro *test capable of predicting these peak plasma concentrations prior to the completion of pharmacokinetic studies, the effect of several classes of anticancer agents against normal human bone marrow myeloid progenitor cells (CFU-GM) was studied. The investigated agents included anthracycline antibiotics, cisplatin and its analogs, anthracene derivatives and two flavone acetic acid derivatives. The CFU-GM were exposed to these agents for* 30–60 min. *An exponential relationship between drug concentration and CFU-GM growth was observed for all compounds with the exception of the flavone acetic acid derivatives which were inactive. For the latter two compounds, an inhibition of CFU-GM growth was observed after continuous exposure. When compared to the plasma concentrations after parenteral administration of these agents, there was a very good agreement between* 1/10 *of the peak plasma concentration and the concentration inducing a* 90% *inhibition of the CFU-GM growth for the anthracycline antibiotics and anthracene derivatives. In contrast, for cisplatin and its analogs, there was a better agreement between* 1/10 *of the peak plasma concentration and the concentration inducing a* 10% *inhibition of CFU-GM growth. The combination of concentrations inducing inhibitions of* 10 *and* 90% *of the CFU-GM growth provides a range of concentrations that predict reasonably well the peak plasma concentrations of several anticancer agents and that could be used as guides for other* in vitro *investigations.*

INTRODUCTION

PROGRESS in the field of cancer chemotherapy has been considerable during the past 25 years. Nevertheless, several common human tumor types remain incurable and there is a clear need for new anticancer agents [1]. The screening process for new anticancer agents relies essentially on the use of murine tumors [2]. The yield of this program has been relatively low and several laboratories have attempted to introduce new screening systems. Among these, the human tumor stem cell assay developed by Salmon and Hamburger [3] as well as human cell lines have been proposed as the primary screening of new compounds [4, 5]. However sensitivity and specificity of these *in vitro* tests rely on the appropriate selection of concentrations and duration of exposure to the new anticancer agent. For anticancer agents for which pharmacokinetic data are available, malignant cells are usually exposed to the peak plasma concentration and

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1/10 of the peak plasma concentration of the drug observed after treatment in human beings [6]. For new compounds, such data are not available at the time of the primary screening. Therefore, presently, arbitrary concentrations such as 10 μg/ml for continuous exposure have been used [4].

The problems created by the selection of arbitrary concentrations for *in vitro* testing of new drugs could be overcome if the peak plasma concentrations of potential anticancer agents could be predicted. In a previous work in which we investigated the sensitivity of normal human bone marrow myeloid progenitor cells (CFU-GM) to anthracene derivatives [7], we reported that a concentration inducing a 90% cell kill was in fact very close to one-tenth of the peak plasma concentrations of these agents. In the present work, we extend our observations to other classes of anticancer agents, including doxorubicin and other anthracycline antibiotics, cisplatin and its analogs, as well as two flavone acetic acid derivatives. The latter two agents were selected because they do not induce myelosuppression in man [8, 9].

MATERIALS AND METHODS

Drug supply

Anthracenedione diacetate (AMT) was supplied by Warner-Lambert Company (Ann Arbor, Michigan). Mitoxantrone (dihydroxyanthracenedione dihydrochloride, MXT) was obtained from Cyanamid Company (U.K.) and bisantrene dihydrochloride (BST) from the American Cyanamid Company (Pearl River, New York). Idarubicin (IDA) and doxorubicin (DOX) were kindly supplied by Farmitalia Carlo Erba (Milano, Italy). Daunorubicin (DNR) was purchased from Rhone-Poulenc (Brussels, Belgium). Menogaril (MEN) was obtained from the Upjohn Company (Kalamazoo, Michigan). Marcellomycin (MCM), carminomycin (CMM), cisplatin (DDP), carboplatin (CBDCA), iproplatin (CHIP), spiroplatin (TNO-6) and ethylene diamine platinum II malonate (NSC 146068, JM-40) were supplied by Bristol-Myers Laboratories (Syracuse, New York). Finally, flavone acetic acid (LM975, NSC 347512) and its diethylaminoester (LM985, NSC 293015) were obtained from Lipha (Lyon, France).

Marrow samples

Four to 5 ml bone marrow samples were obtained from the sternum or posterior iliac crest in healthy volunteers after having obtained informed consent. The samples were anticoagulated with 10 U/ml heparin (Upjohn Company, Kalamazoo, Michigan). Mononuclear cells were separated from the whole marrow sample by Ficoll/Hypaque density centrifugation for 20 min at 800 ***g*** at room temperature. The cells at the interface were collected and washed twice in Dulbecco tissue culture medium and resuspended in the same medium added with 20% fetal calf serum (Gibco, Paisley, U.K.). The yield of this procedure was approx. 3×10^6 mononuclear cells per ml of bone marrow.

Drug testing

All drugs were dissolved in sterile water; MCM was dissolved in water containing 5% dimethylsulfoxide (preliminary experiments did not show any effect of 5% dimethylsulfoxide on CFU-GM growth). The drugs were further diluted in culture medium to obtain the appropriate concentrations; 0.5×10^6 mononuclear marrow cells were incubated for 30–60 min at 37°C in a 2 ml final volume of culture medium containing 20% fetal calf serum and various drug concentrations in a controlled atmosphere containing 7.5% CO_2 and 100% H_2O. For drugs for which pharmacokinetic data were available, concentrations were initially selected from the plasma concentration observed in man after intravenous administration [8–24]. For the drugs for which no pharmacokinetic data were available, arbitrary concentrations (range 0.01–500 μg/ml) were selected. Preliminary experiments further defined the range of concentrations inducing a 0–100% cell kill for each drug. Each drug was tested against 3–14 marrow samples. The incubation was terminated by the addition of a sixfold excess of Dulbecco tissue culture medium at 4°C. Cells were then centrifuged at 800 ***g*** for 10 min. The cell pellets were resuspended in culture medium and cultured as previously described [7].

In separate experiments, bone marrow myeloid progenitor cells were also exposed continuously to LM975 and LM985: the drugs were added to the culture medium at concentrations ranging between 10 and 500 μg/ml.

Data analysis

At least 30 colonies per control plate were required for an experiment to be considered evaluable. Colony counts of the three plates for a particular drug concentration were averaged to obtain one data point; the standard error of the mean for individual data points did not exceed 15% of the mean. To determine cell sensitivity to a particular drug, the percentage of surviving colonies relative to the number of control colonies was plotted vs. drug concentration. The MLAB program [25] was used for semi-logarithmic linear regression analyses. Statistical comparisons were done with a Student's *t* test.

RESULTS

A linear relationship was observed between the logarithm of colony survival and concentration for

*Table 1. Effect of anthracycline derivatives on CFU-GM**

Drug	No. of tests	k	IC_{10}	IC_{50}	IC_{90}
DOX	10	9.4 ± 2.3† (4.3 – 27.0)	0.02 ± 0.01 (0.00 – 0.05)	0.09 ± 0.02 (0.00 – 0.18)	0.34 ± 0.05 (0.10 – 0.55)
DNR	7	24.2 ± 6.3 (6.0 – 60.0)	0.02 ± 0.00 (0.01 – 0.04)	0.06 ± 0.01 (0.03 – 0.11)	0.16 ± 0.04 (0.06 – 0.37)
CMM	7	133.6 ± 44.4 (81.7 – 182.1)	0.001 ± 0.000 (0.000 – 0.003)	0.005 ± 0.001 (0.002 – 0.009)	0.018 ± 0.002 (0.014 – 0.027)
IDA	5	205.6 ± 79.9 (91.5 – 509.0)	0.003 ± 0.002 (0.005 – 0.089)	0.007 ± 0.002 (0.003 – 0.014)	0.019 ± 0.005 (0.006 – 0.029)
MEN	5	6.0 ± 1.1 (2.9 – 9.6)	0.07 ± 0.01 (0.05 – 0.11)	0.19 ± 0.03 (0.12 – 0.28)	0.50 ± 0.09 (0.29 – 0.83)
MCM	14	24.1 ± 4.4 (4.7 – 69.6)	0.01 ± 0.00 (0.00 – 0.03)	0.04 ± 0.00 (0.01 – 0.08)	0.12 ± 0.01 (0.04 – 0.21)
ACL	13	51.5 ± 12.8 (12.7 – 195.3)	0.01 ± 0.00 (0.00 – 0.03)	0.03 ± 0.01 (0.01 – 0.08)	0.08 ± 0.01 (0.02 – 0.20)

*Abbreviations: DOX: doxorubicin; CMM: carminomycin; IDA: idarubicin; MEN: menogaril; MCM: marcellomycin; ACL: aclacinomycin A. k: slope of the best fit curve ((μg/ml)-1); IC_{10}, IC_{50}, IC_{90}: concentrations inducing a 10%, 50% and 90% cell kill, respectively (μg/ml).
†Mean ± S.E.; values in parentheses indicate the range.

*Table 2. Effect of cisplatin and some of its analogs on CFU-GM**

Drug	No. of tests	k	IC_{10}	IC_{50}	IC_{90}
DDP	5	0.059 ± 0.010† (0.035 – 0.086)	1.8 ± 0.6 (0.02 – 3.36)	13.0 ± 2.2 (6.9 – 19.6)	43.9 ± 19.6 (25.6 – 65.0)
CBDCA	5	0.013 ± 0.000 (0.013 – 0.014)	32.9 ± 6.4 (14.2 – 46.1)	77.1 ± 5.5 (60.8 – 88.6)	198.1 ± 3.2 (188.6 – 204.8)
CHIP	5	0.012 ± 0.003 (0.007 – 0.021)	4.3 ± 2.2 (0.0 – 11.9)	52.1 ± 11.2 (32.3 – 85.1)	203.3 ± 35.3 (107.5 – 304.4)
TNO-6	5	1.39 ± 0.34 (0.73 – 2.61)	0.08 ± 0.04 (0.00 – 0.23)	0.58 ± 0.10 (0.30 – 0.87)	1.99 ± 0.37 (0.92 – 2.95)
JM-40	5	0.008 ± 0.005 (0.002 – 0.029)	0.9 ± 0.6 (0.0 – 2.7)	170.5 ± 49.7 (22.4 – 301.3)	661.6 ± 221.1 (78.0 – 1320.0)

*Abbreviations: DDP: cisplatin; CBDCA: carboplatin; CHIP: iproplatin; TNO-6: spiroplatin; JM-40: ethylene diamine platinum (II) malonate. k: slope of the best fit curve ((μg/ml)-1); IC_{10}, IC_{50}, IC_{90}: concentrations inducing a 10%, 50% and 90% cell kill, respectively (μg/ml).
†Mean ± S.E.; values in parentheses indicate the range.

all drugs. The ceofficients of correlation were > 0.990 with only three exceptions (MEN: $r = 0.987$, LM985: $r = 0.988$ and DDP: $r = 0.989$).

In order to evaluate the variability of the effect of each drug, the slope (k) of the best fit curve for the various drugs against each bone marrow sample was calculated as well as the concentrations inducing a 10%, 50%, and 90% cell kill, respectively (IC_{10}, IC_{50}, IC_{90}) (Tables 1–3). The variations in the effect of a given drug against different bone marrow samples are illustrated for marcellomycin (the compound for which the highest number of bone marrows were investigated) in Fig. 1. The ranges and standard deviations from the mean listed in Tables 1–3 indicate the variability for the other compounds.

For the anthracycline antibiotics (Table 1), the difference between DOX and DNR was of borderline significance; there was no significant difference for the IC_{10}, IC_{50} and IC_{90}; the relationship between drug concentration and colony survival was steeper (but not significantly so; $P = 0.29$) for DNR than for DOX. CMM and IDA were clearly more toxic than any other anthracycline ($P < 0.001$ for all comparisons); the relationship beween colony sur-

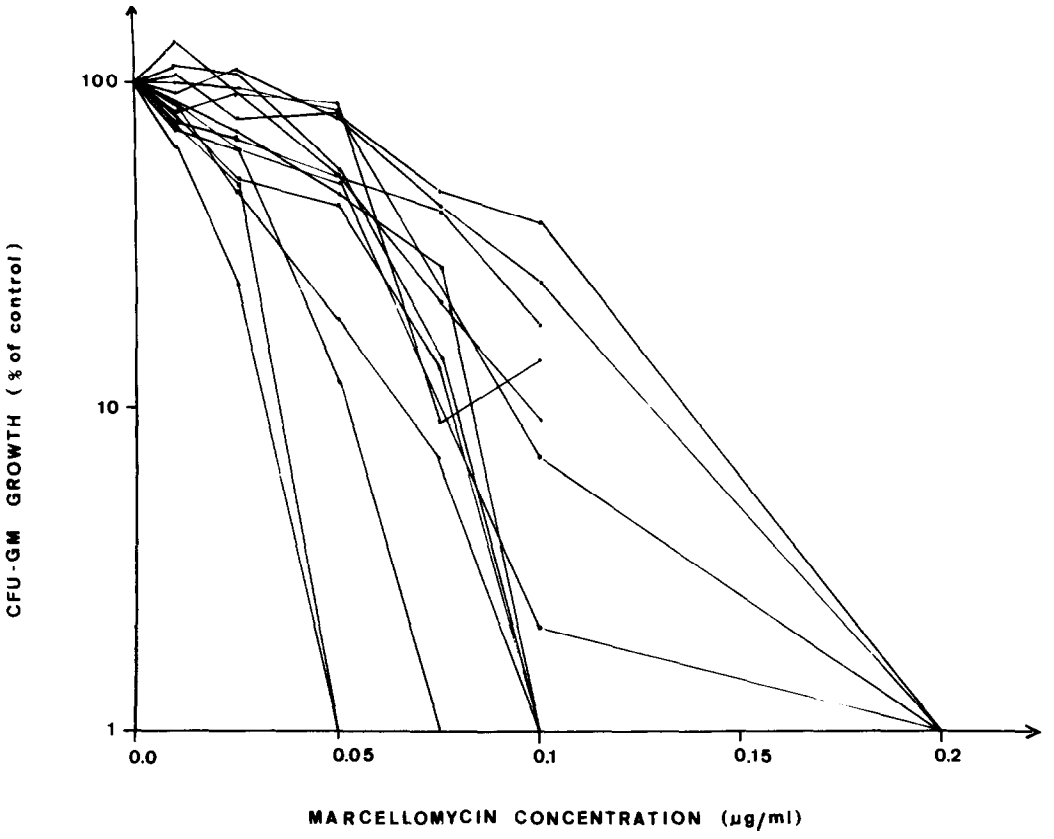

Fig. 1. Effect of marcellomycin on normal human bone marrow progenitor myeloid cells. Each line represent the results obtained with a single bone marrow.

*Table 3. Effect of ametantrone, bisantrene, mitoxantrone, flavone acetic acid and the diethylaminoester of flavone acetic acid on CFU-GM**

Drug	No. of tests	k	IC_{10}	IC_{50}	IC_{90}
AMT	4	3.3 ± 0.9† (0.84 – 4.96)	0.08 ± 0.02 (0.03 – 0.14)	0.36 ± 0.14 (0.17 – 0.77)	1.14 ± 0.52 (0.53 – 2.69)
BST	4	6.9 ± 0.7 (5.91 – 8.82)	0.05 ± 0.02 (0.00 – 0.08)	0.13 ± 0.02 (0.07 – 0.15)	0.37 ± 0.02 (0.33 – 0.42)
MXT	3	229 ± 58 (114 – 278)	0.002 ± 0.002 (0.000 – 0.005)	0.004 ± 0.003 (0.000 – 0.010)	0.012 ± 0.006 (0.005 – 0.024)
LM985	3	0.0055 ± 0.0012 (0.003 – 0.0102)	27 ± 16 (0 – 62)	110 ± 42 (48 – 200)	360 ± 111 (205 – 578)
LM975	5	0.0065 ± 0.0019 (0.0025 – 0.0078)	33 ± 18 (0 – 72)	107 ± 31 (26 – 246)	418 ± 111 (91 – 750)

*Abbreviations: AMT: ametantrone; BST: bisantrene; MXT: mitoxantrone; LM975: flavone acetic acid; LM985: diethylaminoester of flavone acetic acid. k: slope of the best fit curve ((μg/ml)$^{-1}$); IC_{10}, IC_{50}, IC_{90}: concentrations inducing a 10%, 50% and 90% cell kill, respectively (μg/ml).
†Mean ± S.E.; values in parentheses indicate the range.

vival and drug concentration was also much steeper for CMM and IDA than for any other anthracycline derivative. MEN did not differ significantly from DOX and DNR. Finally, both MCM and ACL were significantly more toxic than DOX ($P < 0.001$), with the exception of IC_{10} (difference not significant) and of the slope ($P < 0.022$)). There was no statistical difference between DNR and MCM, DNR and ACL, and ACL and MCM, although the relationship beween colony survival and drug concentration was twice as steep for ACL than for MCM ($P < 0.01$).

For the cisplatin derivatives (Table 2), the slope of the best fit curve was significantly steeper for TNO-6 than for all the other DDP derivatives ($P < 0.001$ for all comparisons). The slope of the best fit curve was steeper for DDP than for CBDCA ($P < 0.001$) and CHIP ($P = 0.021$), but was not statistically different between DDP and JM-40. Similarly, the IC_{10}, IC_{50} and IC_{90} were much lower for TNO-6 than for the other derivatives ($P < 0.001$ for all comparisons); cisplatin was more toxic than CBDCA (P = NS), CHIP ($P \leq 0.036$) and JM-40 ($P < 0.001$ with the exception of the comparison of IC_{90}).

For the anthracene derivatives (Table 3), the slope of the best fit curve was significantly steeper for MXT than for AMT and BST ($P \leq 0.001$ for both comparisons). The difference between AMT and BST was of borderline significance ($P \leq 0.05$). The IC_{10}, IC_{50} and IC_{90} were much lower for MXT than for AMT and BST.

With a 1-h exposure, LM975 and LM985 did not induce any significant inhibition of the CFU-GM. For these two compounds, in another series of experiments, CFU-GM were continuously exposed to the drugs. Significant growth inhibition was observed (Table 3). There was no statistical difference between LM975 and LM985.

*Table 4. Comparison of plasma concentrations and cytotoxic concentrations for CFU-GM**

Drug	1/10 peak plasma concentration		IC_{10}	IC_{90}
DOX	0.1–0.2	(20)	0.02	0.34
DNR	0.1–0.2	(20)	0.02	0.16
CMM	0.002–0.010	(21)	0.001	0.018
IDA	0.02–0.05	(10)	0.002	0.019
MEN	0.01–0.10	(14)	0.07	0.50
MCM	0.1–0.2	(15)	0.01	0.12
ACL	0.07–0.09	(16)	0.01	0.08
DDP	1.0–1.5	(20)	1.8	43.9
CBDCA	3.6–5.4	(21)	32.9	198.1
CHIP	0.5–1.0	(22)	4.3	203.3
TNO-6	0.1–0.2	(23)	0.08	1.99
JM-40	0.5–5.0	(24)	0.9	661.6
AMT	0.4–1.5	(17)	0.08	1.14
MXT	0.005–0.010	(18)	0.002	0.012
BST	0.1–0.2	(19)	0.05	0.37
LM985	0.0–2.5	(7)	27.0	360.0
LM975	200	(8)	33.0	418.0

*Abbreviations: DOX: doxorubicin; DNR: daunorubicin; CMM: carminomycin; IDA: idarubicin; MEN: menogaril; MCM: marcellomycin; ACL: aclacinomycin A; DDP: cisplatin; CBDCA: carboplatin; CHIP: iproplatin; TNO-6: spiroplatin; JM-40: ethylene diamine platinum (II) malonate; AMT: ametantrone; BST: bisantrene; MXT: mitoxantrone; LM975: flavone acetic acid; LM985: diethylaminoester of flavone acetic acid; IC_{10}, IC_{90}: drug concentrations inducing a 10% and 90% cell kill in the CFU-GM assay. Concentrations are expressed in μg/ml.

DISCUSSION

The cytotoxic effect of several anticancer agents on normal human myeloid progenitor cells was studied with the CFU-GM assay. These drugs were selected because of their different dose-limiting toxicity. The dose-limiting toxicity of the anthracycline antibiotics is leukopenia [10, 12, 13, 14, 20, 26, 27]. The dose-limiting toxicity for the anthracene derivatives consists of leukopenia for ametantrone and mitoxantrone and of leukopenia and phlebitis for bisantrene [17, 28, 29]. For the cisplatin derivatives, the dose-limiting toxicity of cisplatin itself is nephrotoxicity whereas it is thrombocytopenia for carboplatin, iproplatin and spiroplatin [20, 23, 30, 31]; the dose-limiting toxicities of JM-40 are nausea, vomiting and nephrotoxicity [24]. Interestingly, with appropriate hydration regimens, cisplatin can be given at higher doses with hematologic toxicity becoming more prominent [32]. Finally, the dose-limiting toxicity of LM985 is neurologic whereas LM975 is still undergoing phase I investigation [8, 9]. Beside the deliberate selection of specific series of anticancer agents, our work was deliberately carried out with a 1 h incubation rather than with a continuous exposure. Since the major and long term aim of our work is the primary screening of new agents, the short incubation has the potential advantage of avoiding drug instability [33]. In addition, most anticancer agents are administered by rapid intravenous injection or short infusion and the 1 h exposure reproduces the pharmacokinetic behavior of these drugs more closely than the continuous exposure.

A single exponential pattern of CFU-GM growth inhibition was observed for all drugs investigated; correlation coefficients of 0.99 or greater were consistent with this type of relationship between drug concentrations and CFU-GM survival. A single exponential relationship between CFU-GM survival and drug concentration suggests good homogeneity in terms of drug sensitivity. Marked individual variations in *in vitro* sensitivity for all drugs were found in our study. These observations are consistent with the variable hematologic tolerance reported clinically with most compounds [10–14, 17, 23, 24, 26, 27, 30–32]. However, comparisons of *in vitro* and *in vivo* data should be interpreted with caution since our *in vitro* studies were conducted with samples from volunteers whereas clinical results were obtained in non-homogeneous populations of cancer patients.

The major aim of this study was to examine to what extent the CFU-GM assay may be useful to predict plasma concentrations and to provide guidelines for other *in vitro* investigations. In Table

4, we compare one tenth of the peak plasma concentration to the IC_{10} and IC_{90} in the CFU-GM assay. For some groups of compounds, the agreement between one tenth of the peak plasma concentration and IC_{90} is excellent; this situation is observed for the anthracycline antibiotics and the anthracene derivatives. Unfortunately, for other important groups of anticancer agents, IC_{90} largely exceeds one tenth of the plasma concentration; for cisplatin, the ratio between the IC_{90} and one tenth of the peak plasma concentration is approx. 30; the corresponding ratio is 50 for carboplatin, 200 for iproplatin and ranges between 130 and 1300 for JM-40. For the cisplatin derivatives, the concentrations inducing a 10% inhibition of the growth of normal bone marrow progenitor cells would be much more appropriate. Our findings are similar to those of Ajani *et al.* [34]; they found that the CFU-GM assay would be useful to select an appropriate concentration for *in vitro* investigation with doxorubicin, but that the test would overestimate the corresponding concentration for cisplatin.

For the flavone acetic acid derivatives, the situation is more complex; in fact, we did not observe any significant inhibition of the CFU-GM growth after 1 h exposure even at very high concentrations (500 μg/ml). For this reason, in another series of experiments, the CFU-GM were continuously exposed to these derivatives. With this continuous exposure, for LM985, the IC_{90} is grossly higher than the plasma concentrations of LM985 observed in man. However, LM985 must be considered as a prodrug of LM975: when LM985 is administered to human beings, negligible plasma concentrations of LM985 are measured whereas concentrations ranging between 10 and 50 μg/ml of LM975 are observed [8]. Phase I and pharmacokinetic studies with LM975 are still ongoing; however, with a 1 h administration, dose-limiting toxicity consisting of body temperature abnormalities was observed at a dose of 6.4 g/m^2 (S. Kaye, oral information). At this dosage, plasma concentrations of approx. 1200 μg/ml have been measured; one tenth of this value falls between the IC_{10} and IC_{90} observed in this study. Thus, the CFU-GM assay appears as capable of predicting plasma concentrations even for compounds with non-hematologic dose-limiting toxicity. Interestingly, the flavone acetic acid derivatives have minimal cytotoxic effect against malignant cells *in vitro* after a short exposure; the major cytotoxic effect is observed with a continuous exposure [35]. Therefore, the CFU-GM assay could also help to select the continuous exposure in case minimal inhibition of the CFU-GM growth is observed with the 1-h exposure even at very high concentrations.

The *in vitro* toxicity on bone marrow CFU-GM cannot be used to predict *in vivo* myelosuppression even when one compares drugs of similar classes. For example, on the basis of our *in vitro* studies, mitoxantrone would be predicted to be approx. 35 times more myelosuppressive than ametantrone whereas the ratio of maximum tolerated dose varies between 10 and 13 [17, 28]; similarly, idarubicin would be predicted to be 10 times more potent than daunorubicin whereas the ratio of maximum tolerated doses is approx. 4 [10, 12]. The failure of the *in vitro* assay to predict for the hematologic toxicity *in vivo* may be related to the large variability of the *in vitro* data. Alternately, incorrect predictions may be related to factors such as biotransformation, tissue distribution and other pharmacokinetic parameters that may vary from one agent to another *in vivo* and that are not taken into account *in vitro*. Finally, the experimental model used in this study may not be the most appropriate to predict for the relative clinical potency of anticancer agents. The precursors that are primarily affected by anticancer agents have not been identified. It is currently impossible to grow cells at a later stage of development than CFU-GM; in contrast, hematopoietic cells at an earlier stage of development may be grown *in vitro* [36]. Whether such cells are more appropriate to predict for the myelosuppressive potential of cytotoxic agents is presently unknown. In any case, the prediction of the CFU-GM assay used in this study is not sufficiently accurate to formulate recommendation for the doses of cytotoxic agents to be administered *in vivo*.

In conclusion, the CFU-GM assay used in this study cannot be used to predict *in vivo* myelosuppression. In contrast, it may provide a relatively accurate range of estimated plasma concentrations; these estimated concentrations could be used for other *in vitro* investigations, prior to the availability of human pharmacokinetic data. A prospective study to validate this concept should be conducted.

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