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Application of human CFU-Mk assay to predict potential thrombocytotoxicity of drugs

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ABSTRACT

Megakaryocytopoiesis gives rise to platelets by proliferation and differentiation of lineage-specific progenitors, identified *in vitro* as Colony Forming Unit-Megakaryocytes (CFU-Mk). The aim of this study was to refine and optimize the *in vitro* Standard Operating Procedure (SOP) of the CFU-Mk assay for detecting drug-induced thrombocytopenia and to prevalidate a model for predicting the acute exposure levels that cause maximum tolerated decreases in the platelets count, based on the correlation with the maximal plasma concentrations (C max) *in vivo*. The assay was linear under the SOP conditions, and the *in vitro* endpoints (percentage of colonies growing) were reproducible within and across laboratories. The protocol performance phase was carried out testing 10 drugs (selected on the base of their recognised or potential *in vivo* haematotoxicity, according to the literature). Results showed that a relationship can be established between the maximal concentration in plasma (C max) and the *in vitro* concentrations that inhibited the 10–50–90 percent of colonies growth (ICs). When C max is lower than IC10, it is possible to predict that the chemicals have no direct toxicity effect on CFU-Mk and could not induce thrombocytopenia due to bone marrow damage. When the C max is higher than IC90 and/or IC50, thrombocytopenia can occur due to direct toxicity of chemicals on CFU-Mk progenitors.

1. Introduction

Megakaryocytopoiesis gives rise to platelets by proliferation and differentiation (endomitosis and cytoplasmic maturation) of specific megakaryocyte progenitors, which are identified *in vitro* as Colony Forming Unit-Megakaryocytes (CFU-Mk). Colonies with as few as three cells reflect the property of megakaryocytic progenitors, and may undergo cycles of endoduplication and maturation in the absence of cell division.

It was not easy to determine the conditions suitable for the routine assay of CFU-Mk, however some protocols have been developed that support their formation *in vitro* by clonal growth and maturation of single progenitor cells. Metcalf et al. (1975) has described the first CFU-Mk culture with murine progenitors from bone marrow on agar-medium. Since identification of megakaryocyte colonies after staining was difficult, Vainchenker et al. (1979) suggested using plasma-clot for obtaining human Mk colonies that can be fixed and stained with good preservation of cell morphology. Other authors showed that plasma-clot is not useful for *in vitro* toxicological studies due to poor reproducibility and the lack of specificity. They suggested the use of a semi-solid matrix, the collagen gel (Dobo et al., 1995; Parent-Massin, 1995b), which could allow clonal growth of megakaryocyte and specific recognition of colonies by fixation of the entire culture and immunocytochemical specific staining.

Thrombocytopenia is defined as a dramatic decrease of platelet counts that may often fall to below 10^9/l and can be distinguished by two main types: toxic drug-induced thrombocytopenia and allergic drug-induced thrombocytopenia (Miescher and Graf, 1980). The first is characterized by direct action of xenobiotics against platelet precursors or against circulating platelets. In the second type, thrombocytopenia is induced by the formation of immune complexes against platelets or by synthesis of auto-antibodies, which react with platelets.

In an attempt to study the first type of thrombocytopenia, a model of megakaryocytic progenitor culture for toxicological investigations was applied by Froquet et al. (2001) to study

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in vitro direct effects of chemicals, however, no study for the optimization and prevalidation of this method has yet been conducted.

Validated *in vitro* haematotoxicity assays have the potential to substantially refine the prediction data derived from animal studies, and also to reduce the number of animals for haematotoxicity testing (Gribaldo et al., 1996; Lewis et al., 1996; Pessina, 1998; Pessina et al., 1999; Gribaldo et al., 1998; Parent-Massin et al., 1993; Parent-Massin and Thouvenot, 1995a; Noble and Sina, 1993; San Roman et al., 1994; Naughton et al., 1992; Schoeters et al., 1995; Van Den Heuvel et al., 1997; Rio et al., 1997; Froquet et al., 2001; Casati et al., 2003).

In this work the predictivity of an *in vitro* assay for *in vivo* human thrombocytopenia, due to a single toxicant exposure, has been investigated. In a previous study it has been demonstrated that the CFU-GM assay is able to predict the exposure levels of xenobiotics that would cause initial neutropenia after acute exposure with a high degree of predictivity, based on the comparison of *in vitro* and *in vivo* data in animals and their transfer to the human model (Pessina et al., 2003). Patients receiving high-dose chemotherapy and autologous bone marrow transplantation frequently acquire a platelet secretion defect (Karolak et al., 1993), as well as a recent phase II study on children receiving chemotherapy for solid tumours, revealed a strong association with grade III/IV dose-limiting thrombocytopenia (Angiolillo et al., 2005). Moreover, another widely used drug like Linezolid has been associated with anaemia and thrombocytopenia, where thrombocytopenia can be a treatment-limiting toxicity (Bernstein et al., 2003).

The aim of this work was to refine and optimize the Standard Operating Procedure (SOP) of the CFU-Mk assay kit of Stem Cell Technology (SCT) in order to detect agents able to induce thrombocytopenia (Stage I) and then to prevalidate the assay for predicting the acute exposure levels that can cause maximum tolerated decreases in the platelets count (Stage II).

During the Stage I of the study the main parameters such as culture medium, drug solvents, source of progenitors, criteria for identification and scoring of CFU-Mk etc., have been defined. Then, *in vivo* data on xenobiotics induced-thrombocytopenia were collected from the literature and used for selecting the drug panel for the next Stage II (prevalidation). The prevalidation, managed as an inter-laboratory study, involved four laboratories (A, B, C, D) was conducted on 10 drugs in accordance with the procedures documented previously by ECVAM in association with experts in this area (Balls et al., 1995; Curren et al., 1995; Balls and Karcher, 1995) and in accordance with pharmacological principles published by the *in vitro* haematotoxicology working group (Gribaldo et al., 1996). For more detailed information on alternative methods and on the phases of the prevalidation processes see also Archer et al. (1997); Blaauboer et al. (1999); Worth and Balls (2001). A prediction model based on the comparison between the *in vivo* maximal concentration in plasma (C max) at therapeutic level and IC values determined *in vitro* was also developed to verify its ability to provide a classification of the risk of thrombocytopenia for each drug exposure.

2. Materials and methods

2.1. Culture media

Collagen gel from the Megacult™-C kit (Stem Cell Technologies, Vancouver) was used as culture medium. Iscove's modified Dulbecco's medium (IMDM), serum free contained 1% bovine serum albumin (BSA), 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 2 mM l-glutamine, 10^{-4} 2-mercaptoethanol, was supplemented with 50 ng/ml of human recombinant thrombopoietin (rh TPO), 10 ng/ml of rh Interleukin-6 (IL-6) and rh Interleukin-3 (IL-3). The final concentration of collagen was 1.1 mg/ml.

2.2. Haematopoietic progenitors

Human umbilical cord blood cells (hu-CBC) from five different donors were supplied frozen by Poietic Technologies (Gaithersburg, MD, USA), according to a protocol approved by the Institutional Review Board (IRB). The cryotubes were stored in liquid nitrogen. A sufficient number of aliquots of each CBC donor were distributed to each laboratory, so that all the participants could work on the same batch of cells during each experimental phase.

2.3. Thawing cells

Cells were rapidly thawed at 37 °C and then diluted with 1 ml of 0.22 μm, filtered solution I, composed of 2.5% human albumin (Fluka, Switzerland) with 5% Dextran 40 (Pharmacia Biotech, Milan, Italy) in IMDM, and 8 ml of 0.22 μm filtered solution II, composed of 10% fetal bovine serum (FBS) (Gibco), with 3 units DNase/ml (Roche Molecular Biochemicals, Basel, Switzerland) in IMDM. The cell suspension was centrifuged at 600*g* for 10 min and the pellet was gently resuspended in IMDM. Cells were then counted in a haemocytometer and diluted to a concentration of 2.3×10^6 cells/ml.

2.4. Drug selection

The main criteria for drug selection were their recognised or potential haematotoxicity, the quality and the quantity of existing *in vitro–in vivo* comparison data. The 10 drugs selected, consisted of 3 antineoplastics (busulfan, 5-FU, BCNU), 1 mycotoxin (T-2 toxin), 1 pesticide (warfarin), 2 anticonvulsants (valproate and carbamazepine), 1 antiulcer agent (cimetidine), 1 antibiotic (D-penicillamine), 1 gout suppressant (allopurinol) (Table 1). With the exception of warfarin all the above drugs are able to induce thrombocytopenia.

All drugs purchased were from single batches, 9 from Sigma–Aldrich (France) and 1, allopurinol, from Glaxo-Wellcome Research and Development, USA. Each laboratory received a Standard Operating Procedure (SOP) and handling instructions for preparing the starting solution of the drugs in water (warfarin, allopurinol), IMDM (BCNU, valproate, cimetidine, D-penicillamine), DMSO (5-FU), acetone (busulfan, T-2 toxin) or methanol (carbamazepine). The final concentration of solvent was 2%.

Table 1
C max values and MW of the chemicals studied.

Chemical	Family	MW	C max (μg/ml)
5-FU	Antineoplastic	130.1	50–300
Busulfan	Antineoplastic	246.3	0.8
BCNU (Carmustine)	Antineoplastic	214.1	1070–1700
T-2 toxin	Mycotoxin (food contaminant)	466.5	4.67–46.6 (ng/ml)
Warfarin	Pesticide/rodenticide	308.3	11.2
Valproate	Antiepileptic	144.2	90
Carbamazepine	Anticonvulsant	236.3	75
Cimetidine	Anti ulcer agent	252.4	500
d-Penicillamine	Antibiotic	149.2	1000
Allopurinol	Gout suppressant	136.1	100

General Note: References for C max
Cancer Chemotherapy Handbook, 2000. Dorr, R.T., Von Hodd, D.D. (Eds.), 2nd ed., Appleton and Lange; Cancer Chemotherapy and Biotherapy (1996). (Chabner, B.A., Longo, D.L. (Eds.), 2nd ed., Lippincott-Raven; The Merck Index. (1983). 10th ed., Rahway, New Jersey, Merck Co., Inc.;
American Hospital Formulary Service - Drug Information 97 (McEvoy, G.K. (Ed.), Bethesda, M.D. American Society of Health-System Pharmacists, Inc. 1997 (Plus Supplements); Goodman and Gilman's The Pharmacological Basis of Therapeutics. 1990. Gilman, A.G., Rall, T.W., Nies, A.S., Taylor, P. (Eds.), 8th ed., Pergamon Press, New York.

2.5. Experimental design

The protocol transfer involved the four laboratories. Each laboratory performed a linearity study (number of cells seeded/colony formation) seeding five different number of cells (5×10^4, 2.5×10^4, 1.25×10^4, 6.25×10^3 and 3.125×10^3). The study required that three experiments be carried out in duplicate.

The protocol performance phase assessed the ability of the SOP to determine the IC of the 10 drugs. It was performed by using 50,000 cells/chamber and was optimised in order to test three drugs simultaneously per experiment per day in duplicate (2 chambers/slide). Each drug was tested three times and for each experiment a different cord blood sample was used. For each drug tested, an internal control (CTRL1 = 5000 cells/chamber; CTRL2 = 50.000 cells/chamber) was used to confirm the proportional relationship between the number of cells cultured and colonies scored.

Ideally, the slope of the line between the number of colonies in CTRL1 and CTRL2 should be close to 1.0 (1:1) and the number of colonies in CTRL2 should be 10 fold higher than the number of colonies in CTRL1.

2.6. Standard Operating Procedure (SOP) for CFU-Mk

The medium, the collagen solution, the test article and the cells were mixed immediately prior to plating the culture slide. According to the experimental design, 11 tubes were prepared containing 950 μl of serum-free medium to which were added 50 μl of cell suspension at 0.23×10^6 cells/ml for control 1 tube (CTRL1), and at 2.3×10^6 cells/ml for the others (2 × CTRL2, 2 × D0; D1–D6).

The tubes were vortexed twice for 5 s, and 600 μl of cold collagen solution were added to each one and then vortexed again twice for 5 s.

32 μl (2% of the final volume) of IMDM (for CTRL1 and CTRL2), 32 μl of vehicles (for D0) or 32 μl of each toxicant dilutions (51x) (for D1–D6) were added to the tubes and the suspensions vortexed again twice for 5 s. Finally, using a 2 ml sterile pipette, 700 μl of the cell medium mixture was dispensed in each well of a previously labelled double chamber slide coated with collagen.

Air bubbles were removed by gently touching bubbles with the end of the pipette and the slides tipped using a circular motion to allow the mixture in the chambers to spread evenly over the surface of the slide.

The slides were placed in a 100 mm petri dish in the presence of an open 35 mm petri dish containing 3 ml of sterile water to maintain optimal humidity during the incubation period. The 100 mm petri dishes were then incubated at 37 °C in air + 5% CO_2.

After 12 days, the collagen was removed (by absorption) and the slides fixed in a methanol–acetone solution (1:3), at room temperature for 20 min for colony fixation. The slides were allowed to air dry for 15 min and were then stored at 4 °C until staining.

As the humidity level during incubation time is critical, each laboratory was asked to check the evaporation rate (ER) of its incubator as previously described (Pessina et al., 2001). Acceptable E.R. values were from 1.1 to 2.5.

2.7. Staining and scoring colonies

The staining procedure was performed according to the MegaCult™-C kit protocol. Briefly, slides were rehydrated with 0.05 M Tris–NaCl buffer (Sigma), pH 7.6 for 20 min, then the buffer was replaced with 0.5 ml of 5% human serum in Tris–NaCl and left for an additional 20 min. 0.5 ml of mouse anti-human GPIIb/IIIa (CD41) antibody (diluted 1:100 in Tris–NaCl containing 5% human serum) was then applied and incubated for 1 h at room temperature and then 0.5 ml of biotin-conjugated goat anti-mouse IgG (diluted 1:150 in Tris–NaCl with 1% bovine serum albumin according to the stock concentration) was added, for 30 min. After washing, 0.5 ml of avidin–alkaline phosphatase conjugate diluted 1:150, was applied and left to incubate for an other 30 min. Slides were then treated with 0.5 ml of alkaline phosphatase substrate solution for 15 min, and counterstained with Evans blue–methanol (1:6) solution for a maximum of 10 min. Evans blue excess was gently rinsed off with distilled water, and slides allowed to air dry. The slides were placed inside a 65 mm gridded tissue culture dish and the colonies scored using an inverted microscope with 20–40X magnification.

CFU-Mk colonies appeared as group of cells that have pink membrane staining with blue nuclei. CFU-Mk colonies range in size from 3 to several hundred megakaryocytes per colony.

Three classes of colonies have been identified according to cell number: small colony (3–19 cells), medium colony (20–50 cells) and large colony (>50 cells). For a correct discrimination between the different types of colonies, it was important to carefully evaluate the number of cells in each aggregate.

To reduce the variability in counting colonies, the laboratories had to follow strictly defined criteria and an internal colony atlas was prepared and circulated. Four types of colonies were included in the counts: *multicentric colonies* with defined shape and with few spread cells on the borders; *multifocal colonies*, the most frequently ones, composed of aggregates of several colonies with or without a peripheral halo of spread cells; *spread colonies*, composed of spread cells without the presence of aggregates; *colonies of platelets* with round shape, a dense central part and a peripheral halo of non-recognizable cells (Fig. 1).

2.8. Statistical analysis

The relationship between the number of cells seeded and the number of CFU-Mk colonies was preliminary studied in the different laboratories by mean of the linear regression analysis and the

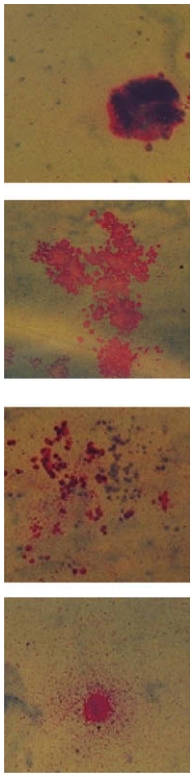

Fig. 1. Morphology of *in vitro* Mk-CFU colonies.

coefficient of determination (R^2) from Pearson coefficient was calculated. From this the optimum cell density (50,000 cells) was chosen. The linearity of CFU-Mk colony formation was verified against the optimum cell density by using a cell density of 5,000 (CTRL1 = 5,000 and CTRL2 = 50,000 cells).

Solvent toxicity on CFU-Mk was verified by comparing the number of colonies found in CTRL2 and that found in D0 (solvent) using the Student *t*-test procedure. For all tests significance was set at $p < 0.05$.

For each drug, the dose–response relationship was analysed using linear regression after log transformation of the number of colonies. For each experiment the values of IC10, IC50 and IC90 were estimated. To compare the variability in the dose–response relation among the different labs the analysis of variance (ANOVA) was used.

2.9. Prediction model

To study the relationship between *in vivo* maximal concentration in plasma (C max) at therapeutic level of each drug and the *in vitro* IC values found in our test has been designed a table of the potential risk predictivity according to four levels:

- *Level 0 (no risk)*: C max is lower than IC10; this means that the chemicals have no direct effect on CFU-Mk and could not induce thrombocytopenia.
- *Level 1 (low risk)*: C max is higher than IC10 but lower than IC50.
- *Level 2 (high risk)*: C max is higher than IC50 but lower that IC90.
- *Level 3 (very high risk)*: C max is higher than IC90.

At level 1, 2, 3, thrombocytopenia is intended as the result of a direct toxicity of chemicals on CFU-Mk progenitors.

3. Results

3.1. Linearity of response

The counts of the absolute number of colonies obtained in each laboratory during the linearity study are reported in Fig. 2. The differential counts of colonies (small, medium, large) are not reported here because did not give (in this study) any additional information. The colonies counted at each cell density vary between the laboratories but the difference is significant only for the laboratory B. As all the laboratories used the same batch of donor cells this different response may be the expression of some specific experimental condition occurred in the laboratory B (e.g. related to cell thaw). However, independently on the absolute counts, the linear correlation between the number of cells cultured (from 3.125×10^3 to 5×10^4) and the number of colonies scored was found to be significant ($p < 0.01$) and very similar in the individual laboratories. The coefficients of determination (R^2) were 0.66; 0.50; 0.63 and 0.83 for the laboratories with a global mean value of 0.61.

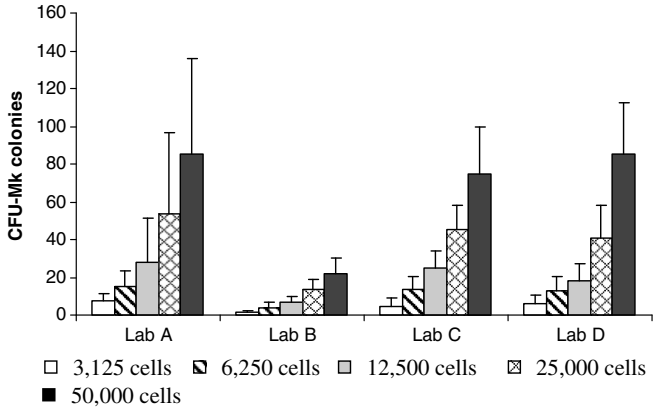

Fig. 2. Counts of the absolute number of colonies (CFU-Mk) in linearity study. In each laboratory, the response in term of number of colonies was evaluated after seeding the following increasing number of cells (see Section 2).

3.2. Protocol performance

The protocol performance phase was designed to test the ability of the assay to reproducibly predict the ICs of the 10 drugs intra- and inter-laboratories. As verified by statistical analyses, the internal linearity control (the main decision criteria for accepting or rejecting experiments) met the acceptance criteria required and consequently, no experiment was rejected.

Since megakaryocyte colony growth has been evaluated in the presence of the solvents used as vehicles for the test compounds, the number of colonies in CTRL2 (in the absence of solvent) and in D0 (with solvent) were compared by a Student *t*-test in order to verify and confirm that solvents (at a fixed concentration of 2%) did not inhibit colony formation. We found that the five solvents used (water, IMDM, DMSO, acetone and methanol) did not modify significantly the CFU-Mk proliferation ($p > 0.05$). For each laboratory the regression curves of test concentration versus the number of CFU-Mk colonies for each drug (performed in triplicate) were used to calculate the mean values of IC10, IC50 and IC90 for each drug.

The analysis performed on data from the four laboratories confirmed a good dose–response correlation and the study by ANOVA tests indicated that, in general, the intra-laboratory variance was

Table 2
Mean ± SD of the IC values.

Drug	IC (μg/ml)	Mean	±SD	N	SEM	SE%
Allopurinol	IC90	310.1(∗)	136.16	10	43.06	13.88
	IC50	103.03	42.48	10	13.43	13.03
	IC10	29.55	11.37	11	3.43	11.60
5-FU	IC90	8.62(∗)	4.67	8	1.65	19.16
	IC50	2.73(∗)	1.40	8	0.49	18.21
	IC10	0.58	0.33	8	0.12	20.31
Busulfan	IC90	4.04	1.58	12	0.45	11.28
	IC50	1.31	0.47	12	0.13	10.34
	IC10	0.32	0.16	12	0.04	14.78
BCNU	IC90	45.41(∗)	25.23	10	7.99	17.57
	IC50	14.62	9.82	10	3.11	21.24
	IC10	3.21	4.15	11	1.25	38.99
Warfarin	IC90	620.60	107.51	12	31.04	5.00
	IC50	198.02	50.60	12	14.60	7.37
	IC10	43.69	32.99	12	9.52	21.79
Valproate	IC90	486.52	225.25	11	67.92	13.96
	IC50	155.41	76.75	12	22.15	14.25
	IC10	22.93	16.32	10	5.16	22.50
Carbamazepine	IC90	81	18.53	12	5.35	6.60
	IC50	26.98	7.84	12	2.26	8.38
	IC10	7.26	4.12	12	1.19	16.40
Cimetidine	IC90	605.30(∗)	374.70	6	152.98	25.27
	IC50	176.23(∗)	114.75	6	46.85	26.58
	IC10	24.65	22.24	7	8.40	34.10
d-Penicillamine	IC90	2351.53(∗)	2270.97	9	756.99	32.19
	IC50	765.60	731.64	9	243.88	31.85
	IC10	235.98	231.16	10	73.10	30.98
T-2 toxin (ng/ml)	IC90	1.09	0.48	12	0.14	12.73
	IC50	0.34	0.17	12	0.05	14.86
	IC10	0.09	0.07	8	0.02	24.87

General Note: (∗) = values obtained by extrapolation on the regression curve out of the range of actual doses tested. N: number of experiments used for the calculation. SEM: Standard Error of Measurement SE%: Standard Error in percentage.

lower than the inter-laboratory one. The inter-laboratory comparisons showed significant differences only for the IC90 determination of 4 out of 10 drugs (allopurinol, BCNU, valproate, T-2 toxin).

In order to estimate the discrepancy between the experimental mean and the true population mean, the IC values of each drug were calculated by using the data from all the experiments independently of the laboratories and therefore expressed as mean +/− s.e.m (standard error of the mean). The results are reported in the Table 2 where the error was also expressed as a percentage of the mean (range from 5% to 38.99%).

3.3. Prediction model

To design a model (presented in Table 4) able to predict the possible risk of *in vivo* thrombocytopenia based on *in vitro* toxicity determined with the Mk-assay the mean values of IC were compared to the maximal plasma concentrations (C max) (see Table 3).

The comparison showed that for warfarin (negative control) the IC10, IC50 and IC90 are higher than the plasma concentration known to induce severe platelet decrease (risk level 0). In effect warfarin is known to induce coagulation disorders in case of intoxication due to its anti-vitamin K effect during haemostasis but no action has been reported on bone marrow progenitors.

For three compounds, T-2 toxin, 5-FU, and BCNU the C max is higher than IC90 (concentration that induces *in vitro* a 90% decrease of megakaryocyte colonies) (risk level 3). For other three drugs, carbamazepine, D-penicillamine, and cimetidine, the C max is lower than IC90 but higher than IC50 (concentration inducing a 50% decrease of megakaryocyte colonies) (risk level 2). In the last three drugs busulfan, allopurinol and valproate, the C max is between IC10 and IC50 (risk level 1). Of course, as C max of allopurinol is much closed to IC50 value (100 versus 103 μg/ml) it could be classified both at risk level 2 or at risk level 1.

Table 3
Comparison between the C max and the IC values.

Drug	C max (μg/ml)	IC Values (μg/ml)		
		IC90	IC50	IC10
Allopurinol	100	310.10	103.03	29.55
5-FU	50–300	8.62	2.73	0.57
Busulfan	0.8	4.04	1.32	0.32
BCNU	1070–1700	45.41	14.62	3.21
Warfarin	11.2	620.60	198.02	43.69
Valproate	90	486.52	155.41	22.94
Carbamazepine	75	81	27	7.26
Cimetidine	500	605.30	176.23	24.65
D-penicillamine	1000	2351.53	765.60	235.98
T-2 toxin	4.67–46.65 ng/ml	1.09 ng/ml	0.34 ng/ml	0.09 ng/ml

Table 4
Suggested prediction model designed on the comparison between C max and IC values.

C max versus IC values	Risk of thrombocytopenia	Drug
C max > IC90	+++	5FU
		BCNU
	(Risk level 3)	T-2 toxin
IC90 > C max > IC50	++	D-penicilamine
		Carbamazepine
	(Risk level 2)	Cimetidine
IC50 > C max > IC10	+	Allopurinol
		Busulfan
	(Risk level 1)	Valproate
C max < IC10	0	Warfarin
	(Risk level zero)	(negative control)

4. Discussion

During haematopoiesis, megakaryocyte progenitors (CFU-Mk) are responsible for platelet production by their proliferation and differentiation. Only mature polyploid megakaryocytes are able to produce platelets by breaking long cytoplasmic extensions in the blood vessels. All the definitions of thrombocytopenia have been based on platelet counts but it can have two origins: a central origin due to bone marrow suppression or a peripheral one, due to damage to the circulating platelets. Both ways can be the consequence of direct toxicity of chemicals on haematopoietic progenitors and/or platelets or of an immune mechanism. Toxicant-induced thrombocytopenia (as toxicant-induced neutropenia) can be described clinically by different parameters such as depth of the nadir (severity), time between exposure to chemicals and nadir, duration of nadir and time to recovery.

To provide a new tool to detect the effect of chemicals on specific haematopoietic progenitors our prevalidation study has been directed to determine if the platelets progenitor (CFU-Mk) clonogenic assay could be used as a predictive test for thrombocytopenia. A clonogenic assay may be useful for thrombocytopenia prediction if it is possible to establish some relationship between the *in vivo–in vitro* concentration able to decrease platelet production or megakaryocyte number. Of course is important to consider that bone marrow is a heterogeneous tissue constituted by different cells such as adipocytes, fibroblasts, monocytes, and haematopoietic progenitors. It is known that lipophilic molecules accumulate in bone marrow adipocytes (Barquet et al., 1981) but no method for measuring concentration of chemicals inside bone marrow is available, so the concentration of xenobiotic in bone marrow is rarely estimated. For this reason, in our prediction model the IC values determined *in vitro* against platelet progenitors (CFU-Mk) have been compared to the value of the maximal concentration in plasma (C max) of the compound being, since this pharmacokinetic parameter is available for humans and plasma is close to the bone marrow compartment.

After the SOP had been refined to obtain optimal performance from the system, a preliminary study on the linearity of colony formation allowed us to setup the best conditions to apply during the study that gave adequate performance in terms of the number of colonies, potential assay sensitivity and linearity. A good intra- and inter-laboratories reproducibility was found suggesting that the optimised protocol could be applied to the prevalidation stage. Finally also a prediction model for estimating *in vivo* thrombocytopenia has been developed.

The main criterion for chemical selection for our study was their capacity to induce thrombocytopenia according to the literature. An analysis of results obtained on each drug, taking into account the toxicity of the drug, the data in the literature, and the different ICs, has been done in order to evaluate the capacity of CFU-Mk assay to predict thrombocytopenia-induced by damage of CFU-Mk progenitors due to a direct toxic effect on bone marrow.

In 3 cases, T-2 toxin, 5-FU and BCNU, the C max is higher than IC90 (corresponding to the concentration which induces a decrease of 90% megakaryocyte colonies). If this dramatic condition is seen *in vitro* at a concentration lower than plasma concentration (that can be the same or more in the bone marrow concentration) it seems possible to argue that platelets numbers can decrease dramatically due to an absence of their production also *in vivo*. In these 3 cases, *in vitro* data are in accordance with *in vivo* data (Young et al., 1999; Karolak et al., 1993; Aymard et al., 1988). For carbamazepine and D-penicillamine, the C max is inferior to IC90 and superior to IC50. This means that the production of megakaryocytes by the bone marrow may decrease by at least 50% as, in effect, is observed in *in vivo* (Sobotka et al., 1990; Kay, 1979). In this class of risk

we have to put also cimetidine although it is known that this drug induces thrombocytopenia by an immune mechanism.

Of course it will be important to validate this model on many drugs and chemicals, however our results show that a relationship can be established between C max and the IC values calculated *in vitro* and this may be used to design a simple prediction model (Table 4).

Of drugs for which the C max is known it is possible to determine *in vitro* IC values to compare with C max. If C max is lower or near to the IC10 value it can be possible to predict the absence of direct toxic effects of the compound on CFU-Mk (that means *in vivo* absence of thrombocytopenia due to bone marrow damage). If C max is "close to"/higher than IC50 and/or IC90, thrombocytopenia can occur (with different risk) due to direct toxicity of chemicals on CFU-Mk progenitors. For new drugs with unknown C max, the CFU-Mk assay is of help in comparing their relative *in vitro* cytotoxicity that is related to their relative potential *in vivo* effects. Furthermore, when entering clinical trials, the dose escalation must be managed with greater caution because plasma levels over IC50 or near to IC90 must be regarded as important threshold risks if they have to be attained *in vivo* for producing therapeutic effects.

Of course many other prediction models (e.g.: based on the correlation between the *in vitro* IC values and other *in vivo* pharmacokinetic parameters as AUC etc.) can be considered and created to verify if they are more efficient than that here proposed.

An interesting observation was that for busulfan, allopurinol and valproate (having C max between IC10 and IC50) only small colonies were scored in the culture (no medium or large colonies were present). This means that since small colonies give rise to a smaller number of megakaryocytes with a low ploidy it may explain the decrease in platelet production. Therefore, CFU-Mk clonogenic assays must be considered a tool with more potential in predicting thrombocytopenia if future studies will incorporate data on the distribution of different sizes of colonies. As megakaryocytopoiesis occurs by endoduplication, the observation of colonies (number and size) can give information on ploidy (a small megakaryocyte has a low ploidy and a big one, a potential 64 N ploidy). On slides, it is possible to determine by observation if they are a majority of small megakaryocytes or big ones. Measure of ploidy on slides obtained using this test could be a new endpoint for future experiments.

Also chemotherapy for solid tumours in both adults and children can be associated with megakaryocyte suppression and ultimately thrombocytopenia as dose-limiting adverse effect. This assay can be used in the pre-clinical phase as well as in early drug development to identify new drugs with no effects on megakaryocytic progenitors maturation, and consequently to reduce thrombocytopenia as dose-limiting risk in phase I patients.

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