Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity

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Although the application of the concept of a threshold to risk assessment is widespread, there remains little experimental evidence for the existence of thresholds for genotoxic compounds, other than aneugens. The clastogenicity of topoisomerase inhibitors is believed to result from the transient stabilization of the topoisomerase enzyme with DNA during the catalytic cycle. This leads to the formation of a stabilized cleavage complex, which, in turn, may result in the formation of a DNA strand break. This indirect mechanism of clastogenicity is the basis for the concept of a threshold for this class of drug. Using micronucleus induction in L5178Y mouse lymphoma cells as a genotoxic end-point, a three pronged approach was used to examine whether the concept of a threshold for clastogenicity could be demonstrated for topoisomerase type II inhibitors in vitro. This involved (i) the study of mechanism (TARDIS assay), (ii) hypothesis testing versus estimation (i.e. scoring up to 10 000 cells/treatment at concentrations immediately above and below the NOEL for micronucleus induction) and (iii) statistical modelling of the concentration–response curves for micronucleus induction. Several topoisomerase type II inhibitors were investigated with varying clastogenic potencies (etoposide < doxorubicin < genistein < ciprofloxacin). Pragmatic thresholds for clastogenicity in L5178Y cells were defined at 0.00236 µg/ml for etoposide, 0.00151 µg/ml for doxorubicin, 1 µg/ml for genistein and 50 µg/ml for ciprofloxacin. In addition, it was demonstrated that etoposide-induced clastogenicity was concentration and time dependent. These results, along with mechanistic data showing that all of the compounds induced concentration-dependent increases in the formation of topoisomerase II stabilized cleavage complexes, provide a weight of evidence to support a threshold concept for clastogenicity with topoisomerase II poisons.

Introduction

Chemicals which interact directly with DNA are thought to abide by the ‘single hit, single target’ hypothesis whereby a mutation may result from a single interaction causing a nucleotide base change (Kirsch-Volders et al., 2000). It is therefore postulated that such chemicals do not exhibit a threshold for genotoxicity. In contrast, for chemicals which cause inactivation or modification of redundant or multiple cellular targets and do not interact directly with DNA the converse is held to be the case, i.e. these chemicals have a threshold dose above which a toxic response is produced. Although the application of a threshold mechanism in genetic toxicology is not new, to date the effect has only been demonstrated experimentally by Elhajouji et al. (1995, 1997) for aneugens. Using a combination of hypothesis testing and estimation (i.e. statistical curve fitting) they examined the threshold concept for chemically induced aneuploidy in human lymphocytes in vitro [determined by micronucleus (MN) induction]. They assumed that when the inflection point is situated between the first statistically significant (FSS) and last non-statistically significant (LNSS) concentrations, then the inflection point would correspond to the threshold which could be estimated by mathematical modelling.

Type II topoisomerases are essential nuclear enzymes found in prokaryotic and eukaryotic cells that regulate the topological state of DNA during replication, transcription and repair. During the topoisomerase II catalytic cycle, the enzyme covalently binds to DNA and produces a temporary double-strand break, thus creating a transient gate (cleavage complex) through which another DNA duplex can pass. After strand passage the break is ligated and the DNA structure is restored. Thus these enzymes perform various DNA cleavage–religation reactions which resolve the torsional stresses created during the processing of DNA (Berger, 1998).

Numerous compounds are known to disrupt the DNA breakage–reunion cycle of mammalian topoisomerase II (Burden and Osheroff, 1998). They may either disrupt the catalytic cycle (catalytic inhibitors) or stabilize the normally transient cleavage complex formed between the enzyme and DNA (topoisomerase II poisons). Whereas catalytic inhibitors disrupt enzyme physiology, topoisomerase II poisons transform the enzyme into a potent cellular toxin through the formation of stabilized cleavage complexes (SCCs) in which the enzyme remains covalently bound to both strands of the DNA double-strand break. Although there is evidence to suggest that the formation of drug-induced SCCs is reversible (Hsiang and Liu, 1998), the disruption of a SCC during DNA transcription or replication will result in DNA strand breaks being exposed and this may lead to clastogenicity and/or cytotoxicity (via apoptosis) if the exposed DNA strand breaks are not repaired (Fortune and Osheroff, 2000). The formation of SCCs can be demonstrated in mammalian cells by the TARDIS (trapped in agarose DNA immunostaining) assay, an immunocytochemical fluorescence technique in which topoisomerase II molecules covalently bound to DNA are localized in situ within the nucleus using specific antibodies (Willmore et al., 1998). Image analysis is used to capture the cell-specific fluorescence intensity and this is used to provide a relative measure of SCC formation within individual cells.

An important implication of an indirect (topoisomerase II-mediated) effect on DNA is the concept of a threshold for clastogenicity (Muller and Kasper, 2000; Sofuni et al., 2000).

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The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) defined an ‘absolute’ threshold as a ‘concentration below which a cell would not ‘notice’ the presence of the chemical’, i.e. a threshold assumes that a biological effect only occurs at concentrations above a certain threshold concentration of a chemical. In other words, ‘the chemical is present but does not effect the cellular target’. However, the precise characterization of such a threshold is difficult to prove experimentally (Schneiderman et al., 1979).

In contrast, a ‘pragmatic’ threshold can be considered as a concentration below which any effect is considered biologically unimportant (Lutz, 1998) and may be defined, in part, with the help of statistical tests (Lovell, 2000). For example, by determining the concentrations at which increases do not exceed the range of responses seen in the negative control in a well-conducted series of experiments. Although a threshold may be defined by statistical modelling, the existence of such a threshold must be justified from biological arguments and not just from statistical ones. Biological mechanisms and effects have to exist which change abruptly or cause a ‘break’ in the process when the ‘effective’ concentration is surpassed (Edler and Kopp-Schneider, 1998).

Therefore, to further examine the threshold concept for topoisomerase type II poison-induced clastogenicity, we selected a battery of compounds with varying clastogenic potencies (etoposide = doxorubicin << genistein << ciprofloxacin) and topoisomerase II affinities. The epipodophyllotoxin etoposide is a highly specific topoisomerase II poison that inhibits the religation stage of the topoisomerase catalytic cycle through the formation of stabilized (drug/topoisomerase/DNA) cleavage complexes (Hsiang and Liu, 1998; Willmore et al., 1998). As a result, etoposide is a potent clastogen, inducing chromosomal damage in vitro and in vivo (Ashby et al., 1994).

Etoposide is also highly genotoxic at the heterozygous thymidine kinase (tk+/-) locus of L5178Y mouse lymphoma cells. DNA sequence analysis of etoposide-induced tk-/- mutants shows that the majority have lost the entire thymidine kinase gene sequence, i.e. 83 and 92% of large and small colony mutants, respectively (Ashby et al., 1994). This is consistent with a clastogenic mechanism of genotoxicity (Applegate et al., 1990).

The anthracycline drug doxorubicin is a topoisomerase II poison that disrupts the topoisomerase II-mediated DNA cleavage–religation reaction resulting in the formation of SCCs (Tewey et al., 1984). In addition to topoisomerase II inhibition, doxorubicin is genotoxic and cytotoxic by other mechanisms, such as intercalation into DNA, free radical formation, lipid peroxidation, DNA adduct formation, DNA cross-linking, effects on DNA strand separation and helicase activity and direct membrane effects (Gerwitz, 1999). However, these other mechanisms are only evident at high concentrations (>1 μg/ml) of doxorubicin and at lower concentrations it is believed that topoisomerase II is the primary target (Cummings et al., 1991; Doroshow, 1996; Gerwitz, 1999). This means that the sole mechanism of clastogenicity at concentrations immediately above the no effect level (NOEL) is likely to be inhibition of topoisomerase II and, therefore, threshold dependent.

The phytoestrogen genistein is a naturally occurring isoflavone found in soy products and is a mammalian topoisomerase II poison (Markovits et al., 1989; Yamashita et al., 1990). Genistein is a competitive inhibitor of tyrosine kinases (Akiyama et al., 1987) and also inhibits growth factor β signalling pathways (Kim et al., 1998) and exerts antiproliferative effects in cells and putative anticancer effects, possibly through the induction of apoptosis (Davis et al., 1998; Shen et al., 2000). Although negative in the Ames test (Bartholomew and Ryan, 1980), genistein is a mammalian genotoxin in vitro (Kulling and Metzler, 1997; Morris et al., 1998) but not in vivo at a dose of 20 mg/kg/day per os for 5 days (Record et al., 1995). The majority of genistein-induced micronuclei in V79 cells were CREST-negative (Morris et al., 1998) and genistein mainly induced small mutant colonies in L5178Y cells (Kulling and Metzler, 1997). These observations suggest that genistein is a clastogen and this is consistent with a topoisomerase II–specific mechanism of clastogenicity.

The targets for quinolone antibiotics are the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (Gootz and Brighty, 1998). Bacterial type II topoisomerases are functionally and structurally related to eukaryotic topoisomerase II enzymes. Many fluoroquinolones are mammalian cell clastogens in vitro (Takahashi et al., 1994; Albertini et al., 1995) and it is believed that mammalian topoisomerase II is inhibited at sufficiently high drug concentrations via the same mechanism as bacteria, i.e. stabilization of the cleavage complex formed between topoisomerase II and DNA (Takayama et al., 1995).

To examine whether the concept of a threshold for clastogenicity could be demonstrated for topoisomerase type II inhibitors in vitro, we have used a three pronged approach and MN induction in L5178Y mouse lymphoma cells as a genotoxic end-point. This involved (i) the study of mechanism (TARDIS assay), (ii) hypothesis testing versus estimation (i.e. scoring up to 10 000 cells/treatment at concentrations immediately above and below the NOEL for MN induction) and (iii) statistical modelling (broken stick analysis) of the concentration–response curves for MN induction. We have reasoned that in the absence of a functional p53 gene, cell death via apoptosis would not be a primary response in L5178Y mouse lymphoma cells and therefore MN induction should more accurately reflect clastogenicity than would be the case in cells with a functional p53, e.g. TK6 cells (where apoptosis may be a confounding factor for threshold modelling). The results of these studies, data analysis and interpretation are presented.

Materials and methods

Cell culture and treatments

TK+/- L5178Y mouse lymphoma cells were maintained in exponential growth phase in RPMI 1640 medium (with 10% v/v horse serum) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), l-glutamine (2 mM), sodium pyruvate (0.5 mM), pluronic acid F68 (0.05% w/v) and HEPES (10 mM). The test compounds were dissolved in dimethyl sulfoxide (DMSO), water or directly into medium. Replicate cultures (6 x 105 cells/ml) were exposed to either chemical or vehicle for 4 h at 37°C. Cells were washed and either reserved for the determination of topoisomerase II stabilized cleavage complex formation (TARDIS assay) or incubated for a further 14 h at 37°C for MN analysis. In addition, relative cell counts were determined to provide an index of cytotoxicity.

Cells were treated with a battery of known mammalian topoisomerase II poisons, i.e. etoposide (100% pure, mol. wt 588.6), doxorubicin (HCl salt, 100% pure, mol. wt 580.0), genistein (98% pure, mol. wt 270.2) and ciprofloxacin (HCl salt, 83.9% as ciprofloxacin, mol. wt 367.8). Formulations of etoposide, doxorubicin and genistein were prepared in DMSO, while ciprofloxacin was prepared in water. All compounds were adjusted for purity and salt form. The vehicle for each compound was used as the negative control. The concentration ranges tested for MN induction were: 0.00001–50 μg/ml etoposide, 0.0002–2 μg/ml doxorubicin, 0.01–50 μg/ml genistein and 0.1–300 μg/ml ciprofloxacin.
In addition, the topoisomerase II poisons ellipticine (99.7% as ellipticine, mol. wt 246.31) and mitoxantrone (HCl salt, 90.6% as mitoxantrone, mol. wt 480.9), the topoisomerase II inhibitor aclarubicin (HCl salt, 93.7% as aclarubicin, mol. wt 848.3) and a clastogen with a non-topoisomerase II mechanism, bleomycin sulphate (93% as bleomycin, mol. wt 1410.4), were used to validate the TARDIS assay. Compounds were adjusted for purity and salt form either in DMSO, water or tissue culture medium. The concentrations used in the TARDIS assay were: 0.04±60 μg/ml etoposide, 13±104 μg/ml genistein, 40±1000 μg/ml ciprofloxacin, 3 μg/ml doxorubicin, 2.6 μg/ml mitoxantrone, 1.2 μg/ml ellipticine, 2 μg/ml aclarubicin and 125 μg/ml bleomycin sulphate, and appropriate concurrent negative controls were included with each experiment. All compounds were purchased from Sigma Chemical Co. (Poole, UK) except for ciprofloxacin (GSK).

The TARDIS (trapped in agarose DNA immunostaining) assay
The assay was conducted as described by Willmore et al. (1998). Briefly, cells were embedded in agarose on microscope slides and subjected to a lysis procedure that removed the cell membrane and soluble proteins. The cells were washed with 1 M NaCl to remove any non-covalently bound nuclear proteins. Topoisomerase II enzymes, covalently bound to nuclear DNA trapped in the agarose, were detected in situ using specific anti-topoisomerase IIa primary antibodies (NuVentures Ltd, Newcastle, UK) and FITC-labelled secondary antibodies (Sigma).

Immunofluorescence images were captured using an epifluorescence microscope and QFish software and nuclear immunofluorescence was analysed using QWin software (Leica Microsystems, Cambridge, UK). Control slide preparations were stained in parallel with alternative primary nuclear antibodies and/or secondary antibodies to exclude non-specific binding or chemical autofluorescence. Nuclear fluorescence was captured (minimum ~100 cells) and corrected for background fluorescence. The median values were calculated for each treatment and these were compared using Kruskal–Wallis and Mann–Whitney statistical tests (with Bonferroni’s correction for multiple comparisons) using Statistica version 5.1 (StatSoft Inc., Tulsa, OK).

Micronucleus assays
Following treatment, multiple slides were prepared (cytocentrifuge) and the cells were fixed in methanol and stained in 0.1 mg/ml acridine orange. MN were scored according to standard cytogenetic criteria (Countryman and Heddle, 1976). All slides were coded and manually scored blind.

Threshold studies
Statistical analysis. Concentration–response curves for MN induction were determined for each compound. The LNSS and FSS concentrations were determined in a stepwise manner using \( \chi^2 \) analysis of the mean MN frequencies. As it is difficult to rule out the possibility of ever smaller but real increases around the NOEL, the number of cells manually scored per
Least squares regression can therefore be used to estimate the maximum concentration above which the per cent MN errors associated with the experimental process, assumed to be independent, were observed, occurs at

\[ \ln(\text{conc.}) = -\beta/2 \gamma \]

Least squares regression can therefore be used to estimate the maximum concentration before cytotoxicity. Above this value, the number of MN begins to fall.

**Broken stick model.** The broken stick model takes the form

\[ \ln(y) = \xi + \gamma \ln(x) + \delta \ln(x)^2 + \epsilon \]

where \( y \) is MN, \( x \) is the concentration at which a particular \( y \) was observed, \( \alpha, \beta \) and \( \gamma \) are the parameters which require estimation and \( \epsilon \) denotes random errors associated with the experimental process, assumed to be independent, normally distributed with constant variance.

Simple calculus shows that the turning point for this curve, the natural logarithm of the concentration at which the maximum number of micronuclei are observed, occurs at

\[ \ln(\text{conc.}) = -\beta/2 \gamma \]

Note that when \( z = 0 \), i.e. the concentration is beneath the threshold value, the broken stick model becomes

\[ \ln(y) = \xi + \gamma \ln(x) + \epsilon \]

while when \( z = 1 \), i.e. when the threshold concentration has been reached, the model becomes

\[ \ln(y) = (\xi + \gamma) + (\omega + \delta) \ln(x) + \epsilon \]

\[ = \gamma' + \delta \ln(x) + \epsilon \]

In other words, the broken stick model implies that, on a log–log scale, a straight line relationship relates ln concentration to ln(MN) before and after the threshold concentration.

It is very much expected that \( \omega \) is statistically insignificant, in which case \( \omega \) is set to 0 as and when appropriate (prior to the change point concentration there is no increase in %MN regardless of concentration applied).

**Time-course study.** Time-course experiments were conducted to determine the effects of exposure (i.e. time and concentration) on MN induction by etoposide. Replicate cultures (6 × 10⁵ cells/ml) were exposed to vehicle or a range of concentrations of etoposide (0.0001–0.025 g/ml) for various times (1–18 h) at 37°C. Cells were washed and incubated for a further 4 h at 37°C for MN analysis. MN induction data were tested for differences between the treatment groups at each time point separately, using a one-way analysis of variance (ANOVA). This was performed on log-transformed data so that the data were more normally distributed. If the ANOVA showed a significant difference between the groups (i.e. \( P < 0.05 \)) then all treatment groups were tested against the control, using a simple \( t \)-test, adjusting for multiplicity using a Bonferroni correction.

**Results**

**TARDIS assay**

The mammalian topoisomerase II poison etoposide induced a statistically significant, concentration-dependent (up to 60 μg/ml) increase in SCC formation in L5178Y mouse lymphoma cells (Figure 1A). Furthermore, upon removal of etoposide (60 μg/ml) from the culture, SCC formation was reversible over time (Figure 1B). Additional studies were conducted with a series of known mammalian topoisomerase II poisons (doxorubicin, genistein, mitoxantrone and ellipticine) and two mammalian non-topoisomerase II poisons (aclacubicin...
and bleomycin). Cytotoxicity was used as a comparator of biological activity in the absence of previous TARDIS assay data in L5178Y mouse lymphoma cells. All of the topoisomerase II poisons except for ellipticine produced statistically significant ($P < 0.001$) increases in SCC formation at concentrations of test compound inducing 50% cytotoxicity. In contrast, there was no change in SCC formation compared with controls for the two non-topoisomerase II poisons, aclacinomycin and bleomycin, at concentrations producing the same levels of cytotoxicity (Figure 1C). Further studies with genistein and the fluoroquinolone antibiotic ciprofloxacin also indicated that both compounds induced statistically significant, concentration-dependent increases in SCC formation in L5178Y mouse lymphoma cells (Figure 1D and E). However, at low concentrations of topoisomerase II poisons, the immunofluorescence signal could not be distinguished from background fluorescence levels in untreated control cells and/or there was no clear concentration–response relationship (data not shown).

**Threshold modelling**

**Etoposide.** The concentration–response data for MN induction for each experiment were combined along with relative cell counts, which provide an index of cytotoxicity (Figure 2A). Etoposide clearly induced statistically significant increases in %MN at concentrations $>0.01 \mu g/ml$ compared with concurrent controls when 2000 cells/culture were scored, although there was virtually no cytotoxicity at this concentration (relative cell counts were 96.8% of controls). Using a combination of hypothesis testing and estimation (i.e. mathematical curve fitting) the threshold concept for etoposide-induced clastogenicity was further examined. The LNSS and FSS concentrations were determined using $\chi^2$ analysis of the mean MN frequencies in a stepwise manner, i.e. by increasing the number of cells scored per culture from 2000 to 10 000 as the NOEL was approached. In cultures where up to 10 000 cells were scored, 0.005 $\mu g/ml$ etoposide induced a statistically significant increase in mean MN frequency compared with the concurrent controls ($P = 0.0145$). In contrast, there was no significant increase at 0.001 $\mu g/ml$ etoposide ($P = 0.3602$). Therefore, it was assumed that the hypothetical threshold lies somewhere between 0.005 and 0.001 $\mu g/ml$ for etoposide. Mathematical modelling was used to estimate the inflection point and define a pragmatic threshold for etoposide clastogenicity in L5178Y mouse lymphoma cells. Under the current study design, the threshold was defined as 0.00236 $\mu g/ml$ etoposide by broken stick analysis with the experimental data accounting for 93.4% of the variability within the model (Figure 2B).

The results of time–course experiments to determine the effects of exposure (i.e. time and concentration) on MN induction by etoposide are shown in Figure 3. There was no statistical difference between groups after 1 h treatment (ANOVA with Bonferroni’s correction, $P = 0.078$) for any of

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**Fig. 3.** Study to determine the effects of exposure (i.e. time and concentration) on MN induction in L5178Y mouse lymphoma cells treated with 0.0001 (grey bar), 0.0010 (pink bar), 0.0025 (blue bar), 0.0100 (red bar) and 0.0250 (magenta bar) $\mu g/ml$ etoposide. $^*P < 0.0166$ ANOVA with Bonferroni’s correction for multiple comparisons.

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**Fig. 4.** (A) Threshold determination using doxorubicin. Blue circle, %MN assay 1; green diamond, %MN assay 2; red square, %MN assay 3. (——) RCC assay 1, (——) RCC assay 2. (——) RCC assay 3. The green line (0.0015 $\mu g/ml$) denotes the LNSS ($P = 0.1972$) and the red line (0.002 $\mu g/ml$) denotes the FSS ($P = 0.0309$). (B) Broken stick model. The breakpoint was identified at ln(conc.) = −6.496 or 0.00151 $\mu g/ml$ on the original concentration scale with ln(%MN) = −0.38 fitted before the breakpoint and ln(%MN) = −5.674 + 0.979 × ln(conc.) fitted afterwards. The goodness of fit ($R^2$) was 89.2%.
the concentrations tested (0.0001–0.025 μg/ml etoposide). There were time-dependent increases in MN induction at 0.010 and 0.025 μg/ml etoposide, with statistically significant increases after 2 h (P < 0.0166). There were also statistically significant increases (P < 0.0166) in MN induction with 0.0025 μg/ml etoposide with ≥8 h exposure (experimental threshold previously defined as 0.00236 μg/ml after 4 h treatment). There were no increases, however, in MN induction at concentrations ≤0.001 μg/ml etoposide (i.e. at concentrations below the defined threshold) for up to 18 h (≥2 cell cycles). These data demonstrate that MN induction by etoposide, above the threshold concentration, is both concentration and time dependent in L5178Y mouse lymphoma cells.

Doxorubicin. The concentration–response data for MN induction by doxorubicin are shown in Figure 4A and the broken stick analysis in Figure 4B. Doxorubicin induced statistically significant increases in %MN at concentrations ≥0.007 μg/ml compared with concurrent controls (2000 cells/culture). In cultures where up to 5000 cells were scored, 0.002 μg/ml doxorubicin induced a statistically significant increase in mean MN frequency compared with the concurrent controls (P = 0.0309). However, there was no significant increase at 0.0015 μg/ml when 10 000 cells were analysed (P = 0.1972). Therefore, the hypothetical threshold was assumed to lie between 0.002 and 0.0015 μg/ml for doxorubicin. Using mathematical modelling the threshold was defined as 0.00151 μg/ml doxorubicin with the experimental data accounting for 89.2% of the variability within the broken stick model (Figure 4B). Thus a threshold for clastogenicity was defined for doxorubicin in L5178Y mouse lymphoma cells at concentrations three orders of magnitude below those reported to be genotoxic by other mechanisms (Gerwitz, 1999).

Genistein. The concentration–response data for MN are shown in Figure 5A. Genistein induced statistically significant increases in %MN at concentrations ≥4 μg/ml (2000 cells/culture), ≥2 μg/ml (5000 cells/culture) and ≥1 μg/ml (10 000 cells/culture) compared with concurrent controls. However, there was no significant increase at 0.5 μg/ml when 10 000 cells were analysed (P = 0.0788). Therefore, the hypothetical threshold was assumed to lie between 0.5 and 1 μg/ml for genistein. Using mathematical modelling the threshold was defined as 1 μg/ml genistein with the experimental data accounting for 88.0% of the variability within the broken stick model (Figure 5B).

Ciprofloxacin. The results of the in vitro MN assays with ciprofloxacin are plotted in Figure 6A, along with relative cell counts. Ciprofloxacin induced statistically significant increases in %MN at concentrations ≥50 μg/ml compared with concurrent controls (2000 cells/culture). However there was no significant increase at 25 μg/ml when 7000 cells were analysed (P = 0.4879). Therefore, the hypothetical threshold lies between 50 and 25 μg/ml for ciprofloxacin. Using mathematical modelling the threshold was defined as 40 μg/ml ciprofloxacin with the experimental data accounting for 77.1% of the variability within the broken stick model (Figure 6B).

Discussion

L5178Y mouse lymphoma cells were treated with various topoisomerase II poisons, etoposide, doxorubicin, genistein and ciprofloxacin. Concentration–response curves were determined for each compound using MN induction as a measure of clastogenicity and relative cell counts as a measure of cytotoxicity.

A combination of hypothesis testing and estimation (mathematical modelling) was used to resolve a 'pragmatic' threshold for clastogenicity for each compound. One of the main issues in defining a 'pragmatic' threshold through low concentration modelling using a biological assay (in this case the in vitro MN assay) is the problem of assay sensitivity (in this case determined by the background MN frequency in the cell line being used). By itself, the definition of a NOEL as the experimental concentration immediately below that which
produces a statistically significant increase (in this case in MN frequency compared with vehicle control) is imprecise and fraught with complications. Hypothesis testing does not take into account statistical variation, the concentration–response curve or the spacing of experimental concentrations (Edler and Kopp-Schneider, 1998). In addition, the sample size is important as it is difficult to rule out the possibility of ever smaller but real increases around the NOEL. Although increasing the sample size can compensate to some degree, it is recognized that hypothesis testing alone does not provide sufficient evidence with which to accept or reject a threshold response. To ameliorate some of these concerns, it was assumed that when the inflection point is situated between the FSS and LNSS concentrations, then the inflection point would correspond to the threshold which could be estimated by mathematical modelling (Elhajouji et al., 1995, 1997).

Therefore, mathematical modelling was used to define the concentration–response curves and, if deemed to be threshold dependent, estimate the breakpoint. Generally, the application of statistical methods to low concentration modelling is based on model rejection (Edler and Kopp-Schneider, 1998) and begins with the simplest model, e.g. linear non-threshold, which if rejected is substituted for progressively more complex models, such as the exponential model or broken stick model, etc. In the current study, a quadratic model with bootstrap simulation was used to estimate the concentration above which the %MN observed was reduced because of cytotoxicity and/or cell cycle inhibition. The resulting concentration was then used to define the upper limit of the data sets to be fitted to the mathematical models used for the estimation of thresholded/non-thresholded clastogenicity. It is important to note that within these delineated data sets, the influence of cytotoxicity (determined by relative cell counts) was minimal, particularly between and including those concentrations defining the LNSS, putative threshold and FSS (Figures 2A, 4A, 5A and 6A). Moreover, the absence of functional p53 activity in L5178Y mouse lymphoma cells suggests that cell death, via apoptosis, is unlikely to be the primary response of the cells to the induction of DNA double-strand breaks. Therefore, for each test compound, the delineated data sets were subjected to exponential (data not shown) and broken stick analysis. The exponential model was rejected for etoposide and doxorubicin based on violations of model assumptions using a number of graphical and numerical procedures (Draper and Smith, 1981). The exponential model could not be fitted to the ciprofloxacin data, whilst mathematical modelling was unable to discriminate between the broken stick and exponential models for genistein ($R^2 = 8$ and $91\%$, respectively). Breakpoint estimates were established for each compound by broken stick analysis and these were observed to fit within the FSS and LNSS concentrations. Thus, ‘pragmatic’ thresholds for clastogenicity in L5178Y cells were defined at $0.00236 \mu g/ml$ for etoposide, $0.00151 \mu g/ml$ for doxorubicin, $1 \mu g/ml$ for genistein and $50 \mu g/ml$ for ciprofloxacin.

These analyses clearly show that thresholds for topoisomerase II inhibitor-induced clastogenicity (determined by MN formation) may be defined by estimation and statistical modelling. The existence of such thresholds, however, must be justified from biological arguments and not just from statistical ones (Edler and Kopp-Schneider, 1998). Therefore, parallel studies using the TARDIS assay (Willmore et al., 1998) were conducted which demonstrated that etoposide, genistein and ciprofloxacin induce concentration-dependent increases in the formation of mammalian topoisomerase II-induced SCC in L5178Y mouse lymphoma cells (i.e. the same cell line used in threshold modelling).

In mammalian cells, the disruption of topoisomerase II/drug-induced SCC by the transcriptosome or DNA replication complex is believed to be the mechanism by which DNA double-strand breaks occur. These, in turn, may result in clastogenicity and/or cytotoxicity (via apoptosis in cells with wild-type p53) if the exposed DNA double-strand breaks are not repaired (Fortune and Osheroff, 2000). Therefore, the interaction of topoisomerase II poisons with DNA (through the
formation of a topoisomerase/DNA/drug cleavage complex) is the biological lesion ultimately responsible for clastogenicity and/or cytotoxicity. It is this indirect mechanism of DNA damage that is generally held for the genotoxicity of topoisomerase II poisons and provides the biological mechanism which underpins the threshold concept. Therefore, the TARDIS studies provide evidence that etoposide, genistein and ciprofloxacin induce the biological lesion ultimately responsible for clastogenicity and thereby support the indirect biological mechanism of DNA damage that underpins the threshold concept for clastogenicity. The observation that etoposide clastogenicity above the threshold (i.e. 0.00236 μg/ml etoposide) is both concentration and time dependent in time-course studies further supports the proposed biological mechanism, as collision events between drug-induced SCC and the transcriptome and/or DNA replication complex would be expected to be time dependent.

The relationship between thresholds for SCC and MN induction was investigated but because of the relatively low sensitivity of the TARDIS assay (due to background fluorescence), no direct correlations could be made with the MN assay (data not shown). It is clear, however, that the relationship between the concentration of topoisomerase II poisons, SCC formation and clastogenicity is complex given the function of time in the ‘collision hypothesis’ described above. Moreover, the reversibility of drug-induced SCC, which is relatively rapid for etoposide (i.e. 80% within 1 h for 60 μg/ml etoposide), suggests that the duration of SCC (which in part may be associated with the affinity of the drug for the topoisomerase II enzyme), will also be an important factor in the probability of DNA double-strand breaks occurring. This can be clearly illustrated using observations with anthracycline-induced SCC formation and clastogenicity. Doxorubicin was the only compound not to induce concentration-dependent increases in SCC in L5178Y mouse lymphoma cells (current study). Similar results have previously been reported for human K562 cells (Willmore et al., 2002). However, in K562 cells the frequency of idarubicin-induced SCC increased with time following removal of the anthracycline drug from the cell culture medium. Furthermore, other groups have observed an increase in DNA strand breaks (resulting from drug-induced topoisomerase II cleavable complexes) after the removal of doxorubicin in the alkaline elution assay (Zwelling et al., 1981; Capranico et al., 1989). Thus, Willmore et al. (2002) concluded that anthracyclines have the ability to induce persistent SCCs that continue to increase DNA damage after drug removal, a property not seen with other topoisomerase II drugs. Nevertheless, there may be a concentration below which the anthracyclines have no discernible biological effect and therefore a ‘pragmatic’ threshold.

In conclusion, we have shown that ‘pragmatic’ in vitro thresholds for clastogenicity can be defined for the topoisomerase II inhibitors etoposide, doxorubicin, ciprofloxacin and genistein in L5178Y mouse lymphoma cells. These results, along with mechanistic data showing that etoposide, ciprofloxacin and genistein induced concentration-dependent increases in the formation of topoisomerase II stabilized cleavage complexes, provide a weight of evidence to support the concept of a threshold for clastogenicity for topoisomerase II poisons. (The genistein TARDIS data and stepwise statistical analysis of MN frequencies on balance support the threshold concept despite the inability of mathematical modelling to exclude a non-thresholded concentration–response relationship.) However, because of the nature of the cell line used (L5178Y mouse lymphoma cells are transformed and p53-deficient), the relevance of these observations to genotoxic risk assessment is uncertain, as are the concentrations which define the ‘pragmatic’ threshold for the topoisomerase II inhibitors used in the present study. The ‘pragmatic’ threshold concentrations may be specific for cell type and the environment of in vitro cell culture (cf. in vivo) and this requires further investigation. Whatever the outcome of future studies, clearly, for chemotherapeutics such as etoposide the therapeutic dose should be significantly above the threshold for clastogenicity/cytotoxicity in order to maximize the cytotoxic effects of the drug on cancer cells. However, for compounds such as genistein, which may have chemotherapeutic effects via (non-genotoxic) cell signalling pathways at physiological doses in vivo, then it is important that the therapeutic dose is below the threshold for clastogenicity in order to avoid mutations which may lead to drug resistance and/or de novo secondary tumours (Baguley and Ferguson, 1998). Similarly, with fluoroquinolone antibiotics it is possible to accept that there may be a threshold of activity for mammalian cells when the affinity of the drug for bacterial gyrase is orders of magnitude higher that it is for mammalian topoisomerase (Muller and Kasper, 2000). As both genistein and ciprofloxacin are clearly clastogenic in vitro but negative in rodents in vivo (Record et al., 1995; Herbold et al., 2001) then the concept of a threshold for clastogenicity for these compounds has important implications for their risk assessment.

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