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Semi-mechanistic description of the in-vitro antiproliferative effect of different antitumour agents

Daniel Moreno, Iñaki F. Trocóniz, Mónica Enguita, Eva Bandrés, Jesús García-Foncillas and María J. Garrido

Abstract

The aim of the present work was to describe the antiproliferative effect of camptothecin (CPT), topotecan (TPT) and cisplatin (CIS) in cultured cells using a semi-mechanistic pharmacodynamic approach. This effect on the growth of DHD-K12PROb cells was modelled as a function of drug concentration and time of exposure using the Gompertz framework. Models reflected two major processes: cell proliferation and cell death/degradation. Antiproliferative effect of CPT and TPT was described as inhibition of cell proliferation, while the effect of CIS was described as stimulation of cell death, including a signal transduction process, reflected as a delay in the onset of drug action. The half-life associated with such a transduction process was estimated to be approximately 27 h. Interestingly, the time profiles of the model predicted a signal transduction process that closely resembled the observed profiles of caspase-3, a protein implicated in CIS-mediated apoptosis. Therefore, the combination of a simple and sensitive design, together with an appropriated modelling strategy, allowed us to explore different mechanisms of action for antitumour agents in cultured cells and to obtain information about the dynamics of signal transduction and the potential use of biomarkers.

Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, Pamplona, Spain

Daniel Moreno, Iñaki F. Trocóniz, Mónica Enguita, María J. Garrido

Laboratory of Pharmacogenomics, Cancer Research Program (Center for Applied Medical Research), University of Navarra, Pamplona, Spain

Eva Bandrés, Jesús García-Foncillas

Correspondence: María J. Garrido, Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, Apartado 177, Pamplona, Spain. E-mail: mgarrido@unav.es

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Introduction

In-vitro studies are a useful way to obtain information about the mechanisms of action of different drugs (Hassan et al 2001). In the cancer area, this type of information has been use to optimize the use of a compound through rational combination with other agents (de Jonge et al 2000) or varying its dosing schedule (Zeghari-Squalli et al 1999).

The ability to induce cell death is related to the mechanism of action of each specific compound. Two types of antitumour agents – topoisomerase I (topo-I) inhibitors, camptothecin and its derivative topotecan, and a DNA alkylator, cisplatin – have been selected to explore the antiproliferative effects in cultured cells. These agents are widely used in the treatment of many human solid tumours (de Jonge et al 2000; Schoemaker et al 2002; Ulukan & Swaan 2002). Camptothecin and topotecan act by forming stabilizing covalent complexes between topo-I and DNA to form a ternary cleavable complex (enzyme–drug–DNA), leading to cell death (Ulukan & Swaan 2002). This principal mechanism of action is S-phase dependent (Feeney et al 2003; Lupi et al 2004). Cisplatin enters the cells and its chloride ligands are replaced by water molecules, generating positively charged hydrated species that react with nucleophilic sites on intracellular macromolecules to form protein, RNA and DNA adducts. These adducts induce the arrest of cells at the G2 phase of the cell cycle, leading to apoptosis (Kartalou & Essigmann 2001).

Several approaches, from in-vitro to in-vivo methods together with empirical or semi-mechanistic mathematical models, have been reported in the literature to study the dependence of cytotoxic drug effects on time and drug exposure (Kalns et al 1995; Guchelaar et al 1998; Gardner 2000; Hassan et al 2001; Lobo & Balthasar 2002). The Gompertz model is a mathematical approach commonly used to describe such dependence, allowing the discrimination between parameters relating to drug, system and experimental design, and in which incorporation of assumptions resembling different mechanistic or/and physiological hypotheses are easy and intuitive (Norton 1988; Simeoni et al 2004). On the basis of these considerations, the purpose of the present work is to characterize the antiproliferative

effect induced by two types of antitumour agents using the Gompertz framework.

Material and methods

Cell culture

The DHD/K12PROb cell line, obtained from a colon adenocarcinoma induced in syngenic BD-IX rats, was used in this study (Segura et al 2004). The cells were grown as adherent monolayers in 25 cm^3 culture flasks at 37°C in a 5% CO_2 humidified atmosphere, and maintained in a mixture of Dulbecco's modified Eagle's and Ham's F-10 medium supplemented with 10% fetal bovine serum and 0.01% gentamicin.

Cells were detached with 0.125% trypsin in EDTA. After trypsinization, cells were counted in a haemocytometer chamber, diluted to appropriate numbers and seeded. In addition, regular assays were performed to eliminate contamination.

Drugs

Camptothecin (Sigma Chemicals, Madrid, Spain), topotecan (generously provided by GlaxoSmithKline, King of Prussia, PA, USA) and cisplatin (Sigma Chemicals) were used for the different treatments.

Camptothecin and topotecan were dissolved in DMSO, and different dilutions were made with 0.9% NaCl to obtain the following final concentrations: 0 (control), 10, 100, 400, 500 and 1000 nM for camptothecin, and 0 (control), 100, 500, 1000, 1500 and 2000 nM for topotecan. Cisplatin was dissolved in 0.9% NaCl and the final concentrations were 0 (control), 2.5, 10, 18, 50, 100 and 200 μM.

Cell growth curves and treatments

Cells were separated into a single-cell suspension in culture medium by trypsinization, and seeded into 96-well culture plates. To evaluate the dependence of drug activity on concentration and exposure time, the plates were seeded with 20×10^3 cells per 180 μL per well and incubated in 5% CO_2 humidified atmosphere at 37°C for 24 h. Each plate was then treated with one of the drugs at different concentrations and incubated for 3, 10, 24, 48 and 72 h. Control cells were grown in the same conditions as the treated plates for 72 h. After each exposure time, the plates were washed twice with sterile phosphate-buffered saline (PBS) (200 μL) and the surviving cells were quantified using the supravital stain neutral red assay (Löwik et al 1993; Weyermann et al 2005).

The cytotoxic effect of 0.1% DMSO (final concentration in each well) at 0, 3, 10, 24, 48 and 72 h was evaluated in the control groups of camptothecin and topotecan, respectively. In addition, to determine the possible loss of cells in the washing procedure, several experiments were developed using one, two, three or four washings with sterile PBS (200 μL) and the loss of cells quantified by the neutral red assay.

Neutral red assay

Neutral red (3-amino-m-dimethylamino-2-methyl-phenazine hydrochloride) (Sigma Chemicals) was used for the identification of vital cells in culture. A stock solution of 1 mg mL^{-1} of neutral red dissolved in double-distilled water was prepared and diluted (1:1) with 1.8% NaCl (working solution) just before use. A volume of 50 μL (working solution) was added to each well and incubated for 1.5 h. After removal, the cells were washed twice with PBS. Finally, the dye was extracted from the cells by the addition of 100 μL 0.05 M NaH_2PO_4 prepared in 50% ethanol. Optical density was read at 540 nm using a microtitre-plate reader (Labsystems iEMS Reader MF, Vantaa, Finland).

In a previous experiment, the relationship between the absorbance measured at 540 nm and different numbers of cells (5×10^3 to 100×10^3 cells per well) was determined.

Measurement of caspase-3 activity

DHD/K12PROb cells (80×10^4 cells per well) were seeded in six-well plates. After 24 h, cisplatin (10, 18, 50 and 100 μM) was added to the medium, following the protocol described above. After each treatment the cells were washed with PBS, harvested by 0.25% trypsin/PBS solution and centrifuged into a pellet. Samples were then incubated with 100 μL lysis buffer at 4°C for 10 min, centrifuged (10 000 *g* for 2 min) and the supernatants stored at −80°C until assay.

The activity of caspase-3 was measured using a commerical caspase-3/CPP32 colorimetric assay according to the manufacturer's recommendations (Biovision MF Mountain View, CA, USA) (Montiel-Duarte et al 2002; Cubedo et al 2006). A standard curve was established with commercial protein.

Data analysis

Data analysis was performed using the NONMEM software, version V (Beal & Sheiner 1992). All data available for each drug were analysed simultaneously.

The time profiles of the number of viable DHD/K12PROb cells (N) in the medium in the absence of the drug were described with equation 1, where dN/dt accounts for the rate of cell change, k_{prol} is the first-order rate constant of cell proliferation and N_{MAX} is the maximum number of cells obtained in the current design conditions, corresponding to 90% of the convergence of the cells in each well. The term $\log (N_{MAX}/N)$ implies zero rate of change when N approximates N_{MAX}:

$$\frac{dN}{dt} = k_{prol} \times \log\left[\frac{N_{MAX}}{N}\right] \times N \quad (1)$$

Drug effect was incorporated as either inhibition of cell proliferation, represented by equation 2:

$$\frac{dN}{dt} = k_{prol} \times (1 - f(C)) \times \log\left[\frac{N_{MAX}}{N}\right] \times N \quad (2)$$

or by stimulation of cell death, represented by equation 3:

$$\frac{dN}{dt} = k_{prol} \times \log\left[\frac{N_{MAX}}{N}\right] \times N - f'(C) \times N \quad (3)$$

where $f(C)$ and $f'(C)$ represent a linear or sigmoidal pharmacodynamic model.

Models integrating both types of drug effect were also fitted to the data.

In general, for short periods of drug exposure, cell death is not immediate and its manifestation requires a certain period of time. Such delay, considered as the time needed to observe the onset of drug action, was implemented using the following expression:

$$\frac{dS}{dt} = k_{del} \times f(C) \times N - k_{del} \times S \quad (4)$$

where dS/dt accounts for the rate of change in a signal transduction process responsible for inducing the antiproliferative effect, and k_{del} is the parameter governing the delay between the time at which the cells begin to be exposed to the drug and the observed onset of action. For the models including the signal transduction process, S substitutes to $f(C)$ or $f'(C)$ at $t=0$, $S=0$.

Selection between models was based on a number of criteria such as graphical analysis of the goodness of fit, precision of parameter estimates and the minimum value of the objective function [−2 log likelihood] provided by NONMEM. The difference in the objective function between the two nested models was compared with a chi-square distribution in which a difference of 3.84 was considered significant at the 5% level.

Statistical analysis

The antiproliferative studies were performed in triplicate. The data represent mean ± s.d. Data were analysed using the non-parametric Kruskal–Wallis test (for more than two groups) or the Mann–Whitney U test (for two groups). Statistical significance was set at $P<0.05$.

Results

The experimental conditions allowed the cells to grow continuously, reaching a plateau 72 h after the start of the experiment. The number of viable cells, which ranged from 5×10^3 to 100×10^3 cells per well, was quantified by a linear curve (0.998) using the absorbance obtained from the neutral red assay.

Control groups in the experiments with camptothecin and topotecan were treated with DMSO 20 μL per well of (0.1% v/v in the culture medium). This concentration of DMSO had no significant effect on cell growth compared with the control group treated with 0.9% NaCl.

Treatment with the antitumour agents induced a concentration-dependent decrease in cell growth, shown in Figure 1. Data from the control experiments had similar profiles, and the variability in the data was comparable across the three drugs.

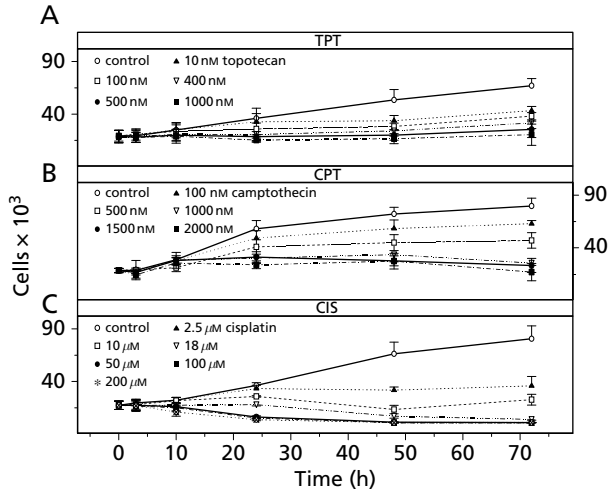

Figure 1 Time course of observed cell viability during treatments with topotecan (top), camptothecin (middle) and cisplatin (bottom). Data represent the mean ± s.d. of three independent experiments; lines correspond to the interpolation of the data.

The time course of viable cells observed in the treatments with topo-I inhibitors and the alkylating agent showed markedly different profiles. Camptothecin and topotecan caused a concentration-dependent decrease in cell viability but none of the curves decreased below baseline. The antiproliferative effect of camptothecin and topotecan was best described as an inhibition of k_{prol}. The model selected for $f(C)$ was an inhibitory E_{MAX} model, where E_{MAX} (the maximum effect elicited by the drug) was estimated with a value very close to 1, indicating that the cell proliferation process can be totally blocked at sufficiently high drug concentration.

Models incorporating a signal transduction process did not improve the fit significantly, and models in which the drugs exerted their effects by stimulating cell death provided a worse description of the data. Lastly, a full model considering drug effect on both types of actions (proliferation and death) was not supported by the data. Figure 2 shows that the model selected provides a good description of the data at all drug concentrations, and at all times of measurement. Table 1 lists the model parameter estimates.

The C_{50} (drug concentration eliciting half the maximum inhibition on k_{prol}) was lower for camptothecin than for topotecan (48.2 nM vs 116 nM), indicating that camptothecin is a more potent inhibitor of cell proliferation.

A model in which the cisplatin effect is incorporated into the stimulation of cell death described the data significantly better than the one considering drug effect at the level of k_{prol} inhibition ($P<0.01$). In addition, the data significantly supported the presence of a signal transduction component ($P<0.01$). For the case of cisplatin, $f(C)$ had the form of an E_{MAX} model. Figure 2 also shows that

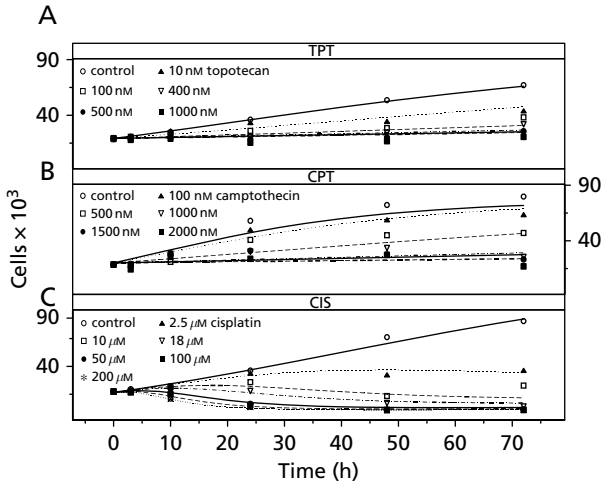

Figure 2 Time course of the observed (symbol) and model-predicted (lines) cell viability for the three antitumour drugs: topotecan (top), camptothecin (middle) and cisplatin (bottom).

Table 1 System and pharmacodynamic parameters for topotecan, camptothecin and cisplatin

	Topotecan	Camptothecin	Cisplatin
N_0 (cells × 10^3)	19.2 (0.03)	20.1 (0.01)	16.7 (0.02)
k_{prol} (h^{-1})	0.024 (0.39)	0.35 (0.15)	0.021 (0.37)
N_{MAX} (cells × 10^3)	94.2 (0.27)	76.5 (0.19)	129 (0.30)
k_{del} (h^{-1})	–	–	0.034 (0.25)
E_{MAX}	0.94 (0.07)	1 (0.02)	0.065 (0.37)
C_{50} (μM)	0.116 (0.51)	0.048 (0.31)	163 (0.37)

N_0, initial number of cells seeded in each well; k_{prol}, first-order rate constant for cell proliferation; N_{MAX}, the maximum number of cells obtained in the experimental conditions; C_{50}, drug concentration eliciting half of the maximum effect (E_{MAX}); k_{del}, parameter controlling the delay of the apoptotic signal. Estimates are listed with the relative standard error in parentheses, calculated as standard error divided by the parameter estimate.

the model selected for cisplatin described the data adequately. The model parameter estimates are listed in Table 1.

Figure 3A represents the model-simulated time profiles of the antiproliferative signal at each concentration of cisplatin studied. It is remarkable that these simulated profiles closely resemble the time course of caspase-3, an intracellular protein directly implicated in cisplatin-mediated apoptosis, measured in a separate experiment (Figure 3B).

Discussion

In the present study the in-vitro antiproliferative effect of two topo-I inhibitors (camptothecin and topotecan) and a DNA alkylating agent (cisplatin) have been described quantitatively in the DHD-K12PROb cell line. This cell line has been reported to be sensitive to topotecan (Segura et al 2004) and cisplatin (Park et al 2002).

The model framework used to analyse our data allowed us not only to discriminate between design, N_0 and N_{MAX}, system- (k_{prol} and k_{del}) and drug-related parameters (C_{50}, E_{MAX}), but also to get indirect evidence of the different mechanisms of action for the two types of anticancer drugs studied. The two topo-I inhibitors exerted their action as an inhibitory effect on the proliferation rate, and the alkylating agent induced cell death.

Our modelling results are supported by literature data. Treatment with 50 nM camptothecin and topotecan for 24 h inhibited the growth of HUVEC cells for 96 h after treatment (Clements et al 1999). Similar results were also observed for IGROV1 cells treated for 1 h with different doses of topotecan (0.05, 0.2, 1, 10 and 100 μM) (Lupi et al 2004). In both studies, the non-cytotoxicity of topotecan was explained as an effect on topo-I inducing an arrest in S-phase. In fact, it has been demonstrated that after repeated low doses of topotecan, the number of cells arrested in the S-phase was higher than with a single high dose, suggesting an over-expression of topo-I resulting from continuous exposure to the drug (Lupi et al 2004).

The time- and concentration-dependent cytotoxic properties of cisplatin have also been reported by other authors with different cell lines (Wu et al 2004; Berndtsson et al 2007). Such behaviour was interpreted as an effect of cisplatin on cell death. A signal transduction process (k_{del}) had to be included to account for the delayed cell response. The signalling pathway associated with cisplatin included apoptosis, a process closely related to the activation of caspase-3 (Cummings & Schnellmann 2002; Wu et al 2004). In fact, this protein seems to play an essential role in the apoptosis mediated by most of the platinum derivatives (Wu et al 2004; Kishimoto et al 2005). We found that the levels of activated caspase-3 were concentration and time dependent (as shown in Figure 3A). Interestingly, the model was able to predict the arbitrary profiles for the hypothetical transduction signal (Figure 3B), which were consistent with the observed data, implicating caspase-3 in the effect of cisplatin and supporting the semi-mechanistic properties of the model.

Unfortunately, these experimental values of caspase-3 could not be included in the model, because the assay used was not sensitive enough to quantify the levels of the protein activated at low doses or for low number of cells after treatments with high concentrations.

Our experimental data show a very steep caspase-3 activation/deactivation profile at high concentrations of cisplatin. This mechanism has been associated with some of the adverse effects of cisplatin, such as renal dysfunction (Wu et al 2004; Berndtsson et al 2007). By contrast, low concentrations of cisplatin elicited smooth caspase-3 activation/deactivation profiles, suggesting that cisplatin might induce tumour cell apoptosis more effectively with continuous exposure to low concentrations (Kishimoto et al 2005).

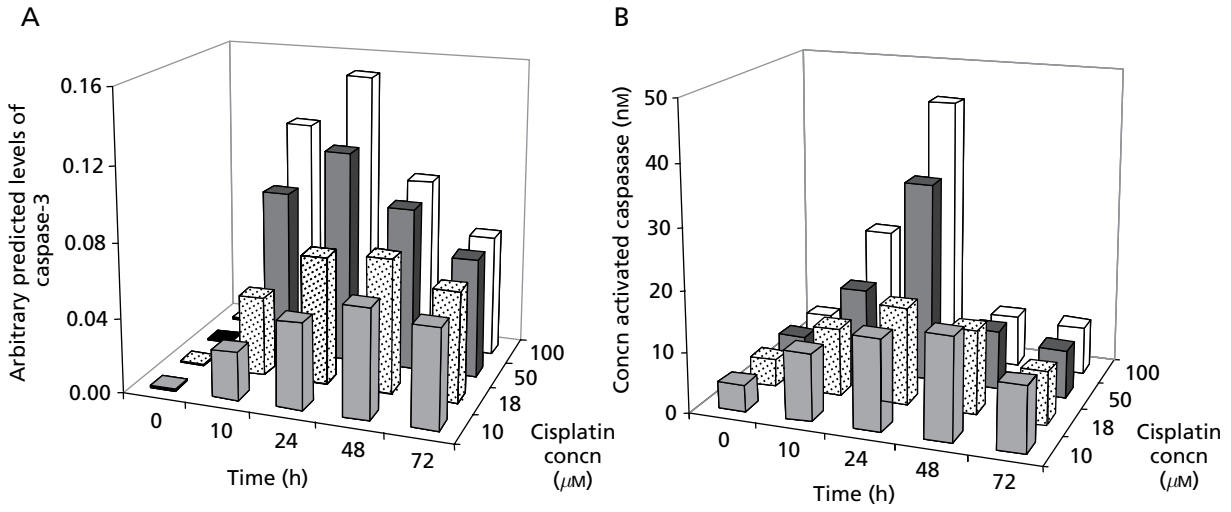

Figure 3 A, Model-based simulated time profiles of the arbitrary levels of the apoptotic signal; B, levels of activated caspase-3, measured after addition of 10, 18, 50 and 100 μM cisplatin at different times of exposure. Bars represent the mean of three experiments.

Conclusion

This paper shows how a simple and sensitive in-vitro experimental design combined with modelling strategies allowed us to explore different mechanisms of action and obtain information about the potential use of biomarkers.

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