Dynamics of cell cycle phase perturbations by trabectedin (ET-743) in nucleotide excision repair (NER)-deficient and NER-proficient cells, unravelled by a novel mathematical simulation approach

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Abstract. *Objectives:* Trabectedin (ET-743, Yondelis[®]) is a natural marine product, with antitumour activity, currently in phase II/III clinical trials. Previous studies have shown that cells hypersensitive to ultraviolet (UV)-rays because of nucleotide excision repair (NER) deficiency, were resistant to trabectedin. The purpose of this study was to investigate whether this resistance was associated with different drug-induced cell cycle perturbations. *Materials and Methods:* An isogenic NER-proficient cellular system (CHO-AA8) and a NER-deficient one (CHO-UV-96), lacking functional ERCC-1, were studied. Flow cytometric assays showed progressive accumulation of cells in G₂ + M phase in NER-proficient but not in NER-deficient cells. Applying a computer simulation method, we realized that the dynamics of the cell cycle perturbations in all phases were complex. *Results:* Cells exposed to trabectedin during G₁ and G₂ + M first experienced a G₁ block, while those exposed in S phase were delayed in S and G₂ + M phases but eventually divided. In the presence of functional NER, exit from the G₁ block was faster; then, cells progressed slowly through S phase and were subsequently blocked in G₂ + M phase. This G₂ + M processing of trabectedin-induced damage in NER-proficient cells was unable to restore cell cycling, suggesting a difficulty in repairing the damage. *Conclusions:* This might be due either to important damage left unrepaired by previous G₁ repair, or that NER activity itself caused DNA damage, or both. We speculate that in UV-96 cells repair mechanisms other than NER are activated both in G₁ and G₂ + M phases.

INTRODUCTION

Trabectedin (ET-743, Yondelis[®]), a tetrahydroisoquinoline alkaloid, is a compound originally derived from the marine tunicate, *Ecteinascidia turbinata* (Rinehart *et al.* 1990; Guan *et al.*

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1993; Jimeno *et al.* 1996) and is currently produced synthetically. This compound shows potent pre-clinical antitumour activity *in vitro* and *in vivo* in several solid tumours such as of the ovary and breast, on melanomas and some sarcomas (Ghielmini *et al.* 1998; Valoti *et al.* 1998; Hendriks *et al.* 1999; Erba *et al.* 2001; Li *et al.* 2001). Phase I and II clinical trials have also shown clinical regression of a range of tumours including soft tissue sarcomas, ovarian cancers (Garcia-Carbonero *et al.* 2004; Sessa *et al.* 2005) and breast cancers (Zeilek *et al.* 2006).

Trabectedin has a unique mechanism of action, alkylating guanine at N2 in the minor groove with some degree of sequence specificity by A and B subunits, causing the major groove to bend towards the minor groove (Pommier *et al.* 1996; Zewail-Foote & Hurley 1999) and interfering with transcription and gene expression (Minuzzo *et al.* 2000). Previous studies have shown that cells in the G₁ phase were more susceptible to the cytotoxic effect of trabectedin than cells in S and G₂ + M phases; however, the main cell cycle perturbation was accumulation of cells in S-late and G₂ + M phases (Erba *et al.* 2001; Martinez *et al.* 2001; Takebayashi *et al.* 2001a).

Trabectedin's cytotoxic effect has been investigated in a variety of cell lines with specific defects in DNA repair mechanisms, and in cells with functional impaired mismatch repair (MMR), resistant to cisplatin were as sensitive to trabectedin as MMR-proficient cells. Cells lacking the catalytic subunit of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) were about three times more sensitive to trabectedin (Damia *et al.* 2001; Erba *et al.* 2001).

A unique response was observed in cells deficient in nucleotide excision repair (NER): trabectedin was four times less cytotoxic in NER-deficient cells than in NER-proficient ones (Damia *et al.* 2001). Takebayashi *et al.* (2001b) and Herrero *et al.* (2006) have reported that trabectedin-resistant cell lines had mutations of XPG (xeroderma pigmentosum, complementation group G) causing impairment of NER. All known DNA-interacting drugs are either more effective or equally effective in NER-deficient cells, so it is still not clear why NER-deficient cells were less sensitive to trabectedin than NER-proficient ones. Because DNA repair systems are activated differently in all cell cycle phases, a thorough analysis of the cell cycle perturbations induced by trabectedin may give clues to the origin of the differences in sensitivity.

The aim of this study was to compare the dynamics of cell cycle perturbations induced by trabectedin on a NER-proficient and an NER-deficient isogenic cell system, by coupling flow cytometry with computer simulation studies. The analysis would follow the cohort of cells being treated with the drug in different cell cycle phases (G₁, S and G₂ + M), thus we would be able to observe different effects of cell cycle perturbations induced by trabectedin, in cells with or without NER.

MATERIALS AND METHODS

Cells and culture conditions

Hamster ovary carcinoma CHO-AA8 (AA8) and its sublines CHO-ERA-5 (ERA-5) and CHO-UV-96 (UV-96) (Damia *et al.* 2001) were grown in monolayer culture in Ham's F-10 medium (Cambrex BioScience, Verviers, Belgium) containing 10% foetal bovine serum (Sigma-Aldrich Co. Ltd., Poole, UK), 1% (v/v) L-glutamine (200 mM), 1% (v/v) HEPES (1 M) and 1.6% (v/v) NaHCO₃ (7.5%) (Cambrex BioScience) at 37 °C, humidified, and with 5% CO₂ atmosphere, in T-25-cm² tissue culture flasks (Iwaki, Bibby Sterilin, Staffordshire, UK). The UV-96 cell line was derived from parental AA8 cells, isolated after treatment with the mutagenic agent azadenine (80 µg/mL). This cell line is UV-hypersensitive because it is defective for ERCC-1, a protein implicated in NER as adaptor of the 5' endonuclease XPF (xeroderma pigmentosum,

complementation group F). The ERA-5 cell line is a clone derived from UV-96 that stably expresses human ERCC-1. AA8, ERA-5 and UV-96 cells all have an estimated doubling time of 13 h.

Drugs

Trabectedin, kindly supplied by PharmaMar (Colmenar Viejo, Madrid, Spain) was dissolved in dimethyl sulfoxide at a concentration of 1 mM, and was kept at -20°C . It was diluted in medium immediately before use.

Growth inhibition and clonogenicity

Exponentially growing cells were treated for 1 or 12 h with different concentrations of trabectedin. They were then washed with phosphate-buffered solution and fresh drug-free medium was added. The growth inhibitory effect of trabectedin and the clonogenicity assay were evaluated by standard methods (Erba *et al.* 2004).

Monoparametric DNA cell cycle analysis

Exponentially growing cells were treated with trabectedin, and at different intervals and after drug washout they were fixed in 70% cold ethanol and kept at 4°C before DNA staining, for cell cycle analysis (Erba *et al.* 2001). Cell cycle phase distribution was calculated as percentages by a Gaussian-modified method (Ubezio 1985).

Biparametric BrdU/DNA analysis

Exponentially growing AA8, ERA-5 and UV-96 cells were treated for 1 h with 20 nM (AA8 and ERA-5) or 80 nM (UV-96) trabectedin. During the last 15 min, $20\text{ }\mu\text{M}$ 5-bromo-2'-deoxyuridine (BrdU) was added to the cells. After treatment, drug-containing medium was removed, cells were washed twice with phosphate-buffered solution and fresh drug-free medium was provided. After 1 h treatment and at intervals after drug washout, the number of control and treated cells was counted and cells were fixed in 70% ethanol and kept at 4°C before staining. This protocol enabled us to obtain a distinct evaluation of the cell cycle perturbations at S phase (BrdU-positive cells), in G_1 or $G_2 + M$ phases (BrdU-negative cells) during treatment. Percentages of G_1 , S and $G_2 + M$ were calculated separately within the BrdU-positive and BrdU-negative subpopulations. Reduced ability of BrdU-positive cells to complete the cell cycle after treatment were quantified by the time course of the fraction of cells originally in S phase, that still remained undivided at a given time (% und), defined as

$$\% \text{ und (t)} = 100 \times \% \text{ BrdU} + \text{und (t)} / (\% \text{ BrdU} + \text{und (t)} + \% \text{ BrdU} + \text{divided (t)}) / 2$$

BrdU/DNA detection

To detect BrdU incorporated into DNA, fixed cells were incubated by using monoclonal anti-BrdU antibody, while DNA was detected using propidium iodide, as previously described (Erba *et al.* 2001).

Computer simulation of cell population growth and drug effects

To combine all these experimental data quantitatively, we used a computer program predicting cell cycle flux in a cell population, starting from the asynchronous cell cycle distribution at the time of treatment. This program has been described in detail elsewhere (Montalenti *et al.* 1998) and has been used in other studies to evaluate behaviour of cells after treatment with different drugs. It is freely available on request to ubezio@marionegri.it. It can reproduce unperturbed

growth of a cell population, constructing a complete and coherent kinetic scenario based on a quantitative estimate of the time- and dose-dependence of the probabilities of cell arrest and killing.

Unperturbed growth

The following inputs are needed to describe the baseline unperturbed growth of control AA8, ERA-5 and UV-96 cells:

- mean transit times in the cell cycle phases T_{G1} , T_S and T_{G2+M}
- intercellular spread of G_1 , S and $G_2 + M$ transit times, measured by the respective coefficients of variation CV_{TG1} , CV_{TS} and CV_{TG2+M}
- initial cell distribution through the cycle phases

Drug effects

To simulate all possible cell cycle perturbations, a set of additional parameters (effect descriptors) was used, each associated with a specific cell cycle response to treatment:

- 1 Delay rate: this is the proportion of cells whose progression inside S phase is inhibited at each step, resulting in a longer mean transit time for this phase. Value of the parameter is equivalent to fractional reduction of average DNA synthesis rate. The extreme situation (delay rate = 1) indicates complete cell freezing in S phase.
- 2 Probabilities of G_1 and $G_2 + M$ block: these are the proportion of cells entering a block in G_1 or $G_2 + M$ phase, instead of proceeding to the next phase. Blocked cells may subsequently either re-enter the cycle or die in the block, depending on the next two parameters.
- 3 Recycling rate: this is the proportion of blocked cells re-entering the cycle, at each time step. It is indicative of recovery of blocked cells and can represent the cells' ability to repair DNA damage.
- 4 Death rate: this is the proportion of cells removed from a group at each time step. Independent death rates can be applied to cycling, blocked or delayed cells in a phase.

The cell cycle model can distinguish first generation cells (present at the time of treatment), second generation cells (after the first division) or successive generations.

The model can calculate percentages of cells in the different generations and in the different phases; the parameters (block, delay and death) may vary according to the generation. If necessary, to reproduce the experimental data more accurately, a beginning time and an end time for the different effects were introduced.

Output data

Providing a set of values of the parameters, as input, describing drug effects (the scenario under evaluation) as output, the simulation program imparts the time course of several measurable quantities. These values are compared directly with the experimental data:

- total number of cells, reproducing the growth curve
- percentages of cells in the G_1 , S and $G_2 + M$ phases
- output of BrdU experiments: percentages of G_1 , S, $G_2 + M$ BrdU-unlabelled cells; percentages of undivided and divided BrdU-positive cells (that is BrdU-labelled cells in the S and $G_2 + M$ phases of their first generation)

Optimization

Hundreds of scenarios were tested by a trial-and-error procedure, starting from the simplest scenario and seeking to fit the experimental data with the fewest parameters maximizing the logarithm of the likelihood function (L):

$$L = \prod_{i,t} (2\pi \sigma_i^2)^{-1/2} \exp(-0.5(x_{i,t} - \text{xfit}_{i,t})^2 / \sigma_i^2)$$

where $x_{i,t}$ refers to the datum of type 'i' at the time 't', $\text{xfit}_{i,t}$ to the corresponding simulated value and σ_i to the standard error for a given measure. Data types were logarithms of absolute cell numbers [$\log(N)$] and flow cytometric percentages. σ_i were 0.2 for $\log(N)$ (equivalent to 20% coefficient of variation of the cell number) and three for cell cycle percentages, corresponding to typical experimental errors for this kind of measure.

Fitting was first attempted keeping parameters constant and equal in the first and second generation. Then, time-dependence and separate values for parameters of first- and second-generation cells were included when required to reproduce the data. Whenever possible, the same parameter values were also used in all cell lines. Similarly, parameters of the cohort of BrdU-positive were kept distinct from those of BrdU-negative cells, when this was necessary.

Likelihood-based 95% confidence intervals for each parameter were obtained by raising or lowering its value until $\log(L)$ was reduced from its best-fit value $\log(L^{\text{best}})$ to the value $\log(L) = \log(L^{\text{best}}) - \chi^2_{0.05,1/2}$ (Aitkin *et al.* 1989).

RESULTS

Clonogenicity assay

Figure 1 shows the effect of trabectedin on clonogenicity of AA8, ERA-5 and UV-96 cells using either a long (12 h, an exposure close to the doubling time of the cell lines) or a short (1 h) drug exposure protocol. AA8 and ERA-5 cells showed similar sensitivity to trabectedin. In the UV-96 NER-deficient cell line, trabectedin was less cytotoxic with IC_{50} between 0.75 and 1 nM in AA8 and ERA-5 and 4 nM in UV-96 cells after 12 h treatment, and between 20 and 40 nM on AA8 and ERA-5 and > 120 nM in UV-96 cells after 1 h treatment.

Cell cycle studies

Monoparametric DNA cell cycle studies

Cell cycle phase perturbations induced by 12 h treatment, at different trabectedin concentrations, were examined after treatment and at different times after drug washout. Figure 2 reports DNA cell cycle analysis performed 12 h after drug washout (left) and cell population growth inhibition after treatment and at different intervals after drug washout (right) (panel A). Trabectedin caused accumulation of NER-proficient AA8 and ERA-5 cells in $G_2 + M$ phase, already detectable at 0.75 nM, and this block was comprehensive at 2 nM. In NER-deficient UV-96 cells, when concentrations causing similar growth inhibition were compared (i.e. 4 and 8 nM), $G_2 + M$ accumulation was not detectable and even at the high concentration it was very low (10 nM). Similar cell cycle phase perturbations were observed after the short (1 h) trabectedin exposure protocol: $G_2 + M$ accumulation was evident only in NER-proficient cells, as shown in Fig. 2, panel B.

BrdU/DNA studies

By labelling cells with BrdU in the last 15 min of drug treatment, we obtained a distinct evaluation of perturbation in cells in S phase (BrdU-positive cells) or in G_1 or $G_2 + M$ phase (BrdU-negative cells) during treatment, using the flow cytometric BrdU/DNA biparametric technique. We performed a BrdU pulse and chase study with AA8, ERA-5 and UV-96 cell lines to compare their proliferation kinetics.

Figure 1. Cytotoxic effects of 1 or 12 h trabectedin exposure on AA8, ERA-5 and UV-96 cell lines. Each point is the mean of five replicates; bar, standard deviation of the mean. Close circles: AA8; close triangles: ERA-5; and open circles: UV-96.

Six measures retrieved from the experiment are shown in the panels of Fig. 3. Overlapping time courses of the absolute cell number and percentage of BrdU in the three cell lines demonstrated that proliferation rates were the same. The kinetics of exit from $G_2 + M$ phase were also the same, as demonstrated by the decrease of percentage of $G_2 + M$ BrdU-negative cells (catching in the first hours only those that were in $G_2 + M$ phase at time zero, when the BrdU pulse was given). Similarly, no differences were observed in the kinetics of exit from G_1 phase, demonstrating that the duration of G_1 was the same, and in the relative movement and percentage of BrdU-positive undivided cells, tracking progression of S-phase cells until they divided. For this reason, a single cell cycle model was built for the three sublines, fitting all data of Fig. 3 together. The best fit model provided the following values of cell cycle durations: T_{G1} 3.5 h; $CV_{T_{G1}}$ 50%; T_S 7 h; CV_{T_S} 20%; T_{G2+M} 2.5 h; $CV_{T_{G2+M}}$ 50%.

To better characterize the different cell cycle phase perturbations induced by trabectedin on NER-proficient and NER-deficient cells, we performed a complete time-course study on AA8 and ERA-5 cells, treated for 1 h with 20 nM, and on UV-96 cells treated with 80 nM, labelling

Figure 2. Panel A: cell cycle perturbation and growth inhibition induced by 12 h exposure to different concentrations of trabectedin. DNA histograms show flow cytometric analysis 12 h after drug washout. Panel B: cell cycle perturbations and growth inhibition induced by 1 h exposure to different concentrations of trabectedin. DNA histograms show flow cytometric analysis 24 h after drug washout.

Figure 3. BrdU pulse-chase experiment of untreated AA8, ERA-5 and UV-96 cell lines. All measures indicate that cell kinetics of the three cell lines was the same. The continuous line shows simulation of the data with a model with T_{G1} 3.5 h, CV_{TG1} 50%, T_S 7 h, CV_{TS} 20%, T_{G2+M} 2.5 h, CV_{TG2+M} 50%, including a temporary (1 h) delay due to manipulation of the flasks for BrdU treatment and washout.

cells with BrdU in the last 15 min of drug treatment. We obtained a distinct evaluation of perturbations of cells in S phase during treatment (BrdU-positive cells), from those in G₁ or G₂ + M phases (BrdU-negative cells).

Figure 4 shows representative BrdU/DNA analysis on AA8, ERA-5 and UV-96 cells, at 0, 2, 4, 8, 12, 18, 24 and 27 h after drug washout. AA8 cells that were in S phase (BrdU-positive cells)

Figure 4. Effect of 1 h trabectedin exposure (20 nM for AA8 and ERA-5 and 80 nM for UV-96 cells) on cell cycle distribution. During the last 15 min 20 μ M BrdU was added to the cells, which were then washed, and drug-free medium was provided. The biparametric BrdU/DNA analysis was performed at different intervals after drug washout. a: control cells; b: trabectedin treated cells.

during drug treatment progressed through this phase of the cell cycle more slowly than control cells. At 2 h after drug washout, only control cells had a fraction of G_1 -positive cells, indicating an impairment of cell division in treated cells. At 4 h after drug washout, those cells treated in G_1 phase (BrdU-negative) were still in G_1 phase. At 8 h after drug washout, most of the control BrdU-negative cells were able to reach S-late/ $G_2 + M$ phase, while the treated cells were still in G_1 /S-early phase.

At 12 h after drug washout, control cells had almost completed one cell cycle. In contrast, the majority of the BrdU-positive trabectedin-treated AA8 cells were in $G_2 + M$ and G_1 phases, while the BrdU-negative cells were in S phase. At 18 and up to 27 h after drug washout, BrdU-positive and BrdU-negative treated cells accumulated in $G_2 + M$ phases. The percentages of $G_2 + M$ cells in NER-proficient cells were 55.8 and 61.9 at 24 and 27 h, respectively.

Trabectedin induced similar cell cycle phase perturbation in ERA-5 and AA8 cells (Fig. 4). In the UV-96 cell line, most BrdU-positive treated cells were in S-late and $G_2 + M$ phases 8 h after drug washout, but no accumulation of cells in $G_2 + M$ phase was observed, even at 12 h. A few of the BrdU-negative treated UV-96 cells were able to exit from G_1 and reach $G_2 + M$ phase, where no clear accumulation was observed. Dot plots shown in Fig. 4 were further analysed to obtain the percentages of cells in each phase, separately for BrdU-positive and BrdU-negative cells.

Simulation of flow cytometric data and cell counts

Instead of trying to interpret each experimental result separately, we used a computer program that simulates the flux of cells in the cell cycle, to explain all data together. Figure 5 shows the absolute cell count obtained by Coulter counter, and percentages obtained by flow cytometry, after 1 h treatment with 20 nM trabectedin in NER-proficient and with 80 nM in NER-deficient cells, together with the best fits (continuous lines) arising from the simulation (control data of each cell line follow the simulation – see Fig. 3 – but were omitted for clarity). Because the differences between the behaviour of AA8 and ERA-5 cells were negligible, the two cell lines were considered together in this analysis. The simulation enabled us to explain together all complex types of behaviour shown in Fig. 5, as a function of few parameters describing the underlying effects of delay, block and recycling (i.e. recovery from block) in each cell cycle phase. Because no dying cells were detected during the observation period (trypan blue exclusion test, data not shown), cell loss was negligible at the concentration used and was not considered in the simulation.

Perturbations in G_1 and $G_2 + M$ phases were described by the percentage of blocked cells, among those passing at a given time the G_1 and $G_2 + M$ checkpoints, respectively, and by the respective recycling rates. Recycling rate is the fraction of previously blocked cells (G_1 or $G_2 + M$) able to re-enter the cycle in the time unit, and measures how fast cells completed processing the damage in G_1 or $G_2 + M$ phase. Delay in S phase was simulated differently, with a parameter describing the reduction of DNA synthesis rate, resulting in a lengthening of average S phase duration.

Additionally, the model distinguishes BrdU-positive from BrdU-negative cells and cells in the first generation (still undivided after treatment) from those that divided (in the second or subsequent generations). Initially, simple hypotheses were tested, assuming the drug effect was restricted to a single phase. All these simple scenarios were found inconsistent with some of the data and were rejected. As we were obliged to include perturbations in all phases of the cell cycle, hundreds of combinations of parameter values were explored.

The complete list of best fit values of the parameters, including their likelihood-based 95% confidence intervals, is reported in Table 1, distinguishing first- from second-generation cells. No perturbations were detected in the third generation. Five to ten percent of the cells exposed

Figure 5. Data (symbols) and best fits (lines) of time courses of absolute cell number and flow cytometric percentages after 1 h trabectedin treatment (20 nM for AA8 and ERA-5 and 80 nM for UV-96 cells). Flow cytometric BrdU/DNA dot plots were analysed and 11 measures were obtained from each of them: percentage of BrdU-positive, undivided BrdU-positive and G_1 , S and $G_2 + M$ of the whole population, percentage of G_1 , S and $G_2 + M$ BrdU-positive cells and percentage of G_1 , S and $G_2 + M$ BrdU-negative cells. Data points represent the average of four (two AA8 and two ERA-5) or two (UV-96) independent experiments. Lines represent best fits obtained from the simulation models of the treatment of AA8/ERA-5 (dotted lines) and UV-96 (dashed lines). As reference, the simulation of untreated cells (continuous line) is also shown.

Table 1. Sensitivity

AA8-ERA5	1st Generation	2nd Generation	UV-96	1st Generation	2nd Generation
G ₁ -block	90% (54.2–100%)	90% (29–100%) [t < 8 h] 0% (0–25.3%) [t > 8 h]	G ₁ -block	90% (70.3–95.4%)	90% (74.1–100%) [t < 8 h] 0% (0–28.2%) [t > 8 h]
G ₁ -recycling	5% (3–7.3%) [t > 2 h]	5% (3–23.5%) [t < 12 h]	G ₁ -recycling	0% (0–1.4%) [t < 4 h] 1.2% (0.9–1.5%) [t < 12 h] 0% (0–0.6%) [t > 12 h]	0% (0–0.3%) [t < 10 h] 1.2% (0.1–1.6%) [t < 18 h] 0% (0–0%) [t > 18 h]
S-delay	40% (23.9–69.9%) [t < 12 h]	40% (5.5–100%) [t < 12 h] 0% (0–33.2%) [t > 12 h]	S-delay	40% (0–65.7%) [t < 12 h]	40% (12.3–41%) [t < 18 h]
G ₂ -block	0% (0–26.9%) [t < 8 h] 75% (65.8–87.6%) [t > 8 h]	75% (65.2–94.5%)	G ₂ -block	0% (0–33%) [t < 8 h] 75% (36.7–86.4%) [t > 8 h]	75% (70–100%) [t < 18 h] 0% (0–94.9%) [t > 18 h]
G ₂ -recycling	0% (0–0.1%)	0% (0–0.2%)	G ₂ -recycling	0% (0–0.2%) [t < 24 h] 0.5% (0–1.6%) [t > 24 h]	0% (0–1.5%) [t < 24 h] 0.5% (0–100%) [t > 24 h]
G ₁ + block	–	90% (0–100%) [t < 8 h] 20% (8.1–26.7%) [t > 8 h]	G ₁ + block	–	90% (39–94%) [t < 8 h]
G ₁ + recycling	–	5% (4–100%) [t < 12 h] 0% (0–1.1%) [t > 12 h]	G ₁ + recycling	–	0% (0–0.6%)
S + delay	30% (0–33.4%) [t < 2 h] 55% (42–56.3%) [t > 2 h]	40% (22–42.5%)	S + delay	70% (69–72.7%) [t < 2 h] 55% (30.3–56%) [t > 2 h]	40% (16.7–51.1%)
G ₂ + block	0% (0–4.3%) [t < 10 h] 95% (23.1–99.2%) [t > 10 h]	95% (89.4–100%)	G ₂ + block	95% (80–97.7%)	0% (0–75.6%)
G ₂ + recycling	1% (0.9–6.6%) [t > 10 h]	0% (0–0.1%)	G ₂ +recycling	0% (0–2.6%) [t < 3 h] 1.2% (1.1–1.9%) [t > 3 h]	–

List of the best fit values of the parameters with 95% confidence intervals within the brackets.

to the drug reached the third generation in the observation period in all the cell lines. BrdU-positive cells were tracked separately from BrdU-negative cells. Moreover, on the basis of time-dependence of the parameters of BrdU-negative cells, it was possible to track separately for a period, events of cells treated in $G_2 + M$ phase from those of cells treated in G_1 phase. Similarly, in NER-proficient cells, the time-dependence of parameters of BrdU-positive cells led to distinction between cells treated in S-early and S-late phases. It should be noted, however, that there was some degree of mixing among cell cohorts moving across these tracks, due to dispersion of residence times in cell cycle phases. This procedure led to the scenarios of trabectedin treatment shown in Figs 6 and 7, for NER-proficient (AA8 and ERA-5) and NER-deficient (UV-96), respectively.

AA8 and ERA-5 cell lines

Cells treated in G_1 and $G_2 + M$ phases (BrdU-negative)

Among BrdU-negative cells, a further distinction can be made between cells treated in G_1 phase and those in $G_2 + M$ phase, at least in the first hours after treatment, because short-time effects in $G_2 + M$ phase are attributable only to $G_2 + M$ -treated cells. Dot plots (Fig. 4) indicated that almost no G_1 -treated cells reached $G_2 + M$ phase less than 8 h from the end of treatment.

The majority of the cells that were in G_1 phase during trabectedin treatment (90% in the best fit shown in Fig. 6) experienced G_1 block. However, they remained blocked only for a short time, as the estimated recycling rate (5% of blocked cells recycling in 0.1 h, the unit of time of the simulation) was equivalent to 2 h in G_1 block (average residence time), after which they entered S phase. Cells were also delayed in S phase, which therefore lasted about 12 h (compared to the 7 h of controls). These cells reached $G_2 + M$ phase after 8 h, but 75% of them experienced a further block there, without any detectable recycling in the 27 h observation period. The remaining 25% traversed $G_2 + M$ phase regularly, divided and no further blocks/delays in G_1 and S phases were detected in the second generation, although a $G_2 + M$ block was detected. No initial $G_2 + M$ block was detected in cells treated in $G_2 + M$ phase: they exited $G_2 + M$ and divided as did control cells. However, their descendants were intercepted by the G_1 block and afterwards their fate was undistinguishable from that of G_1 -treated cells.

Cells treated in S phase (BrdU-positive)

Cells treated in S phase were delayed immediately. However, initially the **average** lengthening of S phase was low, becoming higher after few hours. This suggested different types of behaviour, 'S-late' and 'S-early' cells, the former completing S phase in the first hours after treatment with minor delay, and the latter being delayed more. On the basis of time-dependence of the delay and block parameters, the two cell cohorts were distinguished in the scheme representing the final scenario (Fig. 6).

Cells reaching $G_2 + M$ phase in the first hours after treatment (treated in S-late phase) were not blocked there, divided and were blocked in G_1 phase with fast recycling (or not blocked at all, because the wide confidence interval of this parameters makes this block uncertain), delayed in S phase and comprehensively blocked in $G_2 + M$ phase, similar to $G_2 + M$ -treated cells.

In contrast, cells reaching $G_2 + M$ phase at least 10 h after treatment (cells treated in S-early phase, which had a long S phase) were intercepted by the $G_2 + M$ block already in the first generation. Then, they re-entered in cycle and divided. In their second generation, only a minority of cells of this cohort was blocked in G_1 phase, but their subsequent behaviour (fast recycling, S phase delay and strong $G_2 + M$ block) was not distinguishable from that of cells treated in S-late.

Figure 6. Best-fit model of trabectedin treatment in the AA8 and ERA-5 cell lines. The model distinguishes cell cycle effects in cells that were in G_1 , $G_2 + M$ and S-late or S-early phases during trabectedin treatment. Percentages close to the arrows indicate the cells intercepted by a block (arrows directed to a block compartment), those that were not blocked (arrows from a phase to the following one) or those that re-enter in cycle every 0.1 h (arrows from a block compartment the following phase). Reduction of DNA synthesis rate is indicated in the S-phase rectangles, with the corresponding average durations of the S phase. ND: not detectable.

UV-96 cell line

Cells treated in G_1 and $G_2 + M$ phases (BrdU-negative)

As shown in Fig. 7, almost all UV-96 cells (90%) that were in G_1 phase during trabectedin treatment experienced a G_1 block, as did the AA8 and ERA-5 cells. Differently from NER-proficient cells, however, these UV-96 cells remained blocked for a longer time, as estimated recycling rate

Figure 7. Best-fit model of trabectedin treatment in the UV-96 cell line. The model distinguishes cell cycle effects in cells that were in G_1 , $G_2 + M$ and S phases during trabectedin treatment (see legend Fig. 6).

(1% of blocked cells recycling in 0.1 h) was equivalent to about 9 h in the G_1 block. Then, the cells were delayed in S phase and 75% were intercepted by the $G_2 + M$ block, similarly to AA8 and ERA-5 cells. Yet, in contrast to NER-proficient cells, UV-96 cells seemed able to recycle from the $G_2 + M$ block, although only slowly (0.5% in 0.1 h). The remaining 25% of cells traversed $G_2 + M$ phase regularly, divided and no further blocks/delays were detected in the second-generation period. UV-96 cells treated in $G_2 + M$ phase divided regularly, while their descendants were intercepted by the G_1 block (90%) recycling 1% in 0.1 h. Second-generation cells arriving in S and $G_2 + M$ phases before 18 h were delayed, while thereafter their fate was not distinguishable from G_1 -treated cells, traversing S and $G_2 + M$ phases regularly.

Cells treated in S phase (BrdU-positive)

Cells treated in S phase were comprehensibly delayed immediately. In contrast to AA8 and ERA-5 cells, S-late and S-early UV-96 cells did not behave differently. All S-phase UV-96 cells

behaved similarly to S-early AA8 and ERA-5 cells in the first generation; as they completed the delayed S phase, they were intercepted from the $G_2 + M$ block and then recycled at a slow rate (1.2% in 0.1 h). Differently from AA8 and ERA-5, however, most UV-96 cells were blocked in G_1 phase after division and were unable to recycle up to the end of observation. A minority of cells, bypassing the G_1 block, were delayed in S phase (10 h), while their fate in $G_2 + M$ phase remained uncertain, representing a very low number of cells.

DISCUSSION

We have previously reported that cells deficient in NER were less susceptible to trabectedin treatment than were NER-proficient cells. This was confirmed by our laboratory in a variety of NER-deficient cell lines (Damia *et al.* 2001; Erba *et al.* 2001) and by other laboratories using various cell systems (Takebayashi *et al.* 2001b; Zewail-Foote *et al.* 2001). Now, we have analysed whether trabectedin-induced cell cycle phase perturbations were different in NER-proficient and NER-deficient cells.

We have used an isogenic cell system, NER-proficient CHO cells (AA8), a NER-deficient subclone (UV-96) and a clone of UV-96 cells stably transfected with ERCC-1, the human orthologous gene not functional in UV-96 cells (ERA-5). AA8 and ERA-5 cells were similar in terms of sensitivity to trabectedin and also for cell cycle perturbation caused by the drug, while UV-96 cells were approximately four times less sensitive to trabectedin and showed a different pattern of cell cycle irregularity. It was not possible to compare the cell cycle perturbation at the same concentration of trabectedin, as concentrations that were very cytotoxic in AA8 or ERA-5 cells caused no detectable cytotoxicity or cell cycle perturbation in UV-96 cells. On the other hand, concentration that inhibited growth of UV-96 cells caused complete destruction of AA8 or ERA-5 cells. Therefore, we compared cell cycle perturbation using concentrations that caused comparable growth inhibition, that is, 20 nM in AA8 or ERA-5 and 80 nM in UV-96 cells. Concentrations of trabectedin used in this study to perform biparametric BrdU/DNA flow cytometric analysis coupled with computer simulation studies, were chosen from prior sets of experiments where different trabectedin concentrations were compared among the three different cell lines, to obtain similar growth inhibition rates with no cell death. In particular, we tested the effects induced by 1 h exposure by using 10, 20, 40 and 80 nM trabectedin in all the three cell lines. In addition, cell cycle phase perturbation induced by trabectedin on AA8, ERA-5 and UV-96 cells using different concentrations were tested in all sets of experiments performed.

Standard flow cytometric assays indicated that trabectedin caused a much greater accumulation of cells in $G_2 + M$ in AA8 or ERA-5 cells than in UV-96 cells. In order to elucidate the dynamics of this cell cycle perturbation, we applied a recently developed computer simulation method, which defines the intensity (percentage of cells) and duration of the blocks, as well as the exit rate from the blocks in a quantitative manner (Lupi *et al.* 2004, 2006).

In all three cell lines, the cells treated in either $G_2 + M$ or G_1 were first intercepted at the G_1 checkpoint. However, in AA8 and ERA-5 cells the recycle rate from G_1 block was much faster (5% of cells in 0.1 h) than in UV-96 cells (1% in 0.1 h). It is conceivable that some trabectedin adducts are processed rapidly in AA8 and ERA-5 cells by NER mechanisms, thus allowing these cells to exit the G_1 phase. Because NER is not functional in UV-96 cells, these had necessarily to use other mechanisms to deal with the trabectedin-induced DNA damage

(Hoeijmakers 2001; Friedberg 2003), which caused a prolonged stay in G_1 phase, as demonstrated by our analysis. However, in AA8, ERA-5 and UV-96 cells, DNA repair in G_1 phase was only partial, as indicated; once cells exited G_1 phase, S-phase progression was much slower than in untreated cells (11.6 h S-phase duration compared to 7 h for untreated control cells). Moreover, when they reached $G_2 + M$ phase, 75% of the cells were again intercepted by the $G_2 + M$ checkpoint. The $G_2 + M$ block was irreversible (within the 27 h observation period) in AA8 or ERA-5 cells, but UV-96 cells start exiting from the block in the last hours of observation.

Thus, NER activity in G_1 phase may possibly be dominant over other forms of repair in G_1 phase in AA8 and ERA-5 cells, and it also triggered a signal to make cells exit G_1 phase rapidly. However, this NER activity in G_1 phase evidently was not very efficient in repairing trabectedin damage, as cells were comprehensively delayed in their further progression in S phase, and specially, in G_2M phase. In these phases, other repair mechanisms may try to manage residual trabectedin-induced damage or the additional damage produced by incomplete NER activity. Among these, homologous recombination repair, an S-late/ $G_2 + M$ -specific mechanism that is accurate and error-free, is likely to be activated during long-term $G_2 + M$ block (Valerie & Povirk 2003).

In contrast, UV-96 cells, not having a functional NER mechanism, already activated other repair mechanisms in G_1 phase, slower than NER, as indicated by the lower G_1 -recycling rate. However, they were more efficient than NER, as the residual damage, left unprocessed in cells exiting G_1 phase, was processed faster and more easily in $G_2 + M$ phase in 80 nM-treated UV-96 cells (with detectable $G_2 + M$ recycle) than in 20 nM-treated AA8 and ERA-5 cells (without detectable $G_2 + M$ recycle). Interestingly, all lines of $G_2 + M$ -treated cells were not intercepted by the $G_2 + M$ checkpoint immediately after treatment, but only in the second generation. The most probable explanation in our opinion is that triggering the $G_2 + M$ checkpoint (i.e. completing the cascade of events leading to the block) required some time from the start of treatment and in the meantime cells could complete $G_2 + M$ phase (2.5 h) then divide.

When the analysis focused on cells that were in S phase during trabectedin exposure, we found that their cell cycle progression, after reaching G_1 phase, was similar to that of cells treated in G_1 and $G_2 + M$ phases, but more differences were detected between AA8 or ERA-5 and UV-96 cells, in the way they completed their first cycle after treatment. Immediately after treatment, we could distinguish a difference in the behaviour of cells in S-late or in S-early phase only in AA8 and ERA-5, not in UV-96 cells. AA8 or ERA-5 cells that were at the end of S phase during drug treatment were not blocked in the subsequent $G_2 + M$ phase and divided without detectable delay, like cells treated during the $G_2 + M$ phase. Instead, S-early AA8 or ERA-5 cells were strongly delayed crossing the S phase, and were blocked in $G_2 + M$ phase for a while before dividing. S-late UV-96 cells too were delayed and blocked in $G_2 + M$ phase, like S-early cells. It is not clear why the distinction was evident for AA8 or ERA-5 and not for UV-96 cells. However, concentration of trabectedin used for AA8 and ERA-5 was one-quarter of that used for UV-96 cells. It is possible that S-late AA8 and ERA-5 cells did not have enough time to sense the relatively lower trabectedin-induced damage, to activated S-phase delay and $G_2 + M$ block before division. Alternatively, a high proportion of the relatively low number of DNA adducts occurring at the end of S phase might have been rapidly repaired by NER, and the residual damage was below the threshold that activates the $G_2 + M$ checkpoint. When cells were at the beginning of S phase, adducts formed an obstacle to the normal mechanism of DNA synthesis in both NER-proficient and NER-deficient cells, as demonstrated by the slow S phase, that lasted approximately double that of controls. It is possible that trabectedin DNA adducts not only

reduced the rate of DNA synthesis, but also led to errors and alteration of normal DNA structure, that was then recognized by the sensors of DNA damage in $G_2 + M$ and G_1 phases. Then, S-phase-treated AA8, ERA-5 and UV-96 cells showed differences in their behaviour after division, in the second generation. Thus, rapid recycling from G_1 phase was observed in NER-proficient but not in NER-deficient cells, like in cells treated in G_1 and $G_2 + M$ phases. On exit from G_1 phase, these cells carried out a slow S phase and eventually were blocked in $G_2 + M$ phase. In contrast, UV-96 cells, which had all experienced strong S-phase delay and $G_2 + M$ block in the first generation, were also blocked in G_1 after division.

Taken together, this analysis suggests three main differences in the response to trabectedin damage, of NER-proficient AA8 or ERA-5 and NER-deficient UV-96 cells:

- fast recycling from G_1 block in NER-proficient cells, slow recycling in NER-deficient cells
- no immediate delay of NER-proficient cells treated in S-late phase, but strong delay in NER-deficient cells
- no recycling from $G_2 + M$ block in 27 h in NER-proficient cells, but slow recycling in NER-deficient cells

That cells with functional NER appear to overcome the G_1 block more easily, but then are blocked in $G_2 + M$ phase, suggests that the NER mechanism itself leads to some sort of DNA damage. Perhaps in the presence of trabectedin adducts the repair is incomplete or error prone, thus ultimately amplifying DNA damage caused by the drug, instead of achieving DNA integrity, as expected from a DNA repair mechanism. Certainly, other repair mechanisms make up for lack of NER in UV-96 cells and appear to be much more efficient, as they allow these cells to tolerate 20 nM of trabectedin, without any cell growth inhibition, while AA8 or ERA-5 proliferation would be strongly reduced.

Independently of NER status of the cells, the G_1 phase seems an important checkpoint for trabectedin's activity: cells treated in G_1 phase were affected in crossing this cell cycle phase, and cells treated in S-late or $G_2 + M$ phases were perturbed when they reached G_1 phase. Behaviour of S-early cells, which had just started to synthesize DNA, seemed very similar to that of G_1 cells.

In conclusion, resistance to trabectedin of cells deficient in NER appears to be associated with differences in the cell response to DNA damage, leading to different kinds of cell cycle perturbation. DNA repair mechanisms other than NER are presumably involved, counteracting trabectedin-induced DNA damage more effectively. The simulation model we used to interpret dynamics of cell cycle perturbations provided much more information than a simple illustration of the data. For example, in both cell lines there were cell cycle blocks, recovery and progression of the cycle, followed by further blocks. Most literature on cell cycle checkpoints supports the paradigm that after DNA damage cell cycle checkpoints are activated, causing transient arrest mainly in G_1 or $G_2 + M$ phase, which allows the cell to repair the damage, restoring its integrity before moving on to S phase or mitosis, respectively. This could be a mechanism that protects the cells from synthesizing DNA or undergoing cell divisions when there is still damage in the genome. The present study indicates that the cell response to DNA damage, at least after exposure to trabectedin, is much more complex, as cells appear to be able to overcome blocks in progression through the cell cycle, even when damage is not completely repaired, as indicated by further cell cycle perturbation and blocks later. We cannot even exclude that activation of repair machinery causes a generic delay in the cell cycle, with only a loose association with specific cell cycle phases.

Future studies will be designed to elucidate whether, and to what extent, repair mechanisms other than NER are involved in dealing with trabectedin-induced DNA damage, and how such mechanisms may be related to the dynamics of cell cycle perturbation, which have now been elucidated in much more detailed than in previous studies.

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