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Use of plasma cytotoxic activity to model cytotoxic pharmacodynamics of anticancer drugs*

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Summary. We have developed a pharmacokinetic/pharmacodynamic approach that integrates the disposition, cytotoxic activity and interaction of anticancer drugs. Fundamental to this approach is the measurement of the cytotoxicity, against a "target" cell line, of patient plasma collected at different times after administration of the anticancer agent(s). To illustrate this approach, we have studied the plasma cytotoxic activity (PCA), against HL-60 cells, of plasma from 11 acute myeloblastic leukemic patients treated with daunorubicin (DNR). Plasma, obtained before and serially for 24 h after DNR treatment, was assayed by HPLC for DNR and daunorubicinol (DNRol), its active metabolite. The corresponding observed PCA values (PCA_{obs}) against HL-60 cells were also measured with a flow-cytometric cell-survival assay that we had developed previously. The pharmacodynamics, i.e. PCA, were co-modeled (dual Hill equation with an interaction term to allow synergism or antagonism) with the pharmacokinetics. The integration of the PCA profile provided the area under the observed PCA versus time curve (AUC_{obs}). For each patient, we also generated an "interaction panel", by adding known amounts of DNR and DNRol to his or her pretreatment plasma. The corresponding cytotoxicities were measured, and then applied to the pharmacodynamic model. This provided a standard surface from which the PCA of each sample obtained after therapy was predicted (PCA_{prd}), on the basis of assayed concentrations of DNR and DNRol in that sample. For plasma samples obtained after treatment, the model simultaneously fit all three outputs, i.e. PCA and DNR/DNRol concentration, very well. We observed substantial interpatient variability in HL-60 growth rate in medium containing patient pretreatment plasma, in DNR activity in pretreatment plasma, and in the in vitro activity (PCA) of plasma obtained after DNR treatment. We also compared the AUC_{prd} to the AUC_{obs} for each patient, and we identified a subset of 4/11 acute myeloblastic leukemic patients who had developed much more PCA after DNR administration than could be explained by the measured concentrations of DNR and DNRol. This may be due to unidentified active metabolites or to factors produced in the plasma in response to the treatment. This pharmacokinetic/pharmacodynamic model is promising to describe pharmacodynamics and interactions of anticancer drugs in cancer patients.

Introduction

Resistance to chemotherapeutic drugs at the level of the neoplastic cell is, deservedly, an area of current, intense and extensive research. However, it is conceivable that the efficacy of an administered chemotherapeutic drug (or combination) may also be influenced by different parameters that may be completely independent of tumor cell resistance to drugs. These parameters may be host-specific and may include (a) the extent of metabolism of the drugs to other active or inactive species; (b) the binding to macromolecules (e.g. plasma proteins), distribution and clearance of the administered drug and its metabolites; (c) the dynamic interactions (e.g., synergism or antagonism) between parent drug and metabolites and other administered drugs and their metabolites; (d) the release by the host, in response to drug administration, of factors that may modulate drug activity. These parameters may differ among

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Table 1. Patient characteristics

Patient	Sex/age (years)	DNR dosage (mg m^{-2} day^{-1})	Type of leukemia[a]	Stage of disease[b]	Previous chemotherapy[c]	Liver dysfct.[d]	Renal dysfct.[d] BUN/Cr[e]	Response[f]
1	F/32	45	M_4EO	ND	No	No	No	CR
2	F/34	45	M_5	ND	No	No	35/3	CR
3	F/47	45	Biphenotypic	Relapse of erythroleuk.	Ara-C/DNR AMSA	No	47/2.9	NE
4	M/58	45	CML	Blast crisis	Hydroxyurea	No	No	NR
5	M/59	45	M_2	ND	No	No	No	NE
6	F/57	45	M_1	ND	No	No	No	NR
7	M/77	30	M_1	ND	No	No	No	NE
8	M/66	45	M_2	MDS	No	No	69/4.0	NE
9	F/63	30	M_4	ND	No	No	No	CR
10	F/51	45	M_1	Relapse*	Alkylator HD Ara-C/ASP	No	No	CR
11	F/67	30	M_6	ND	No	No	No	CR

[a] Classification by the French-American-British (FAB) scheme
[b] ND, newly diagnosed; MDS, history of myelodysplastic syndrome; *, leukemia secondary to therapy for ovarian carcinoma
[c] Ara-C, cytosine arabinoside; AMSA, *m*-amsacrine; HD, high dose; ASP, asparaginase
[d] Dysfct. dysfunction
[e] BUN, blood urea nitrogen (mg/dl); Cr, serum creatinine (mg/dl)
[f] CR, complete remission; NE, non-evaluable; NR, no response

patients and may explain some of the variability observed among patients in their clinical outcome [4, 9, 12].

Exposure of cultured tumor cells to chemotherapeutic drugs in vitro gives an estimate of neoplastic cellular sensitivity to the parent compounds, but because of the parameters mentioned above, such data may not reflect the total cytotoxic effects of the drugs when they are administered to a cancer patient. For these reasons, new pharmacodynamic approaches for studying and predicting, in vitro, the behavior and the cytotoxicity of anticancer drugs when administered to humans are needed.

We have developed a pharmacodynamic/pharmacokinetic approach that, in theory, may be applied to integrate the overall impact of the factors mentioned above. Fundamental to this approach is the measurement of the plasma cytotoxic activity (PCA), against a "target" cell line, of patient plasma collected at multiple time points following the administration of an anticancer drug. In addition, when appropriate analytical chemical methods exist, the concentrations of parent drug and known metabolites or other factors known to affect the activity of the drug are measured in each plasma sample and related to the corresponding PCA. This method may be applied to compare the PCA observed in the plasma, obtained during anticancer therapy, to the predicted cytotoxicity, measured when the parent drugs, metabolites or factors thought to be involved in the total activity of the drug, are added to pretreatment plasma of the same patient. Within an individual, a significant discrepancy between observed and predicted PCA might then suggest that factors not yet identified may be present. In theory, the method may be useful to explore, in vitro, the in vivo pharmacodynamic interactions of a drug and its plasma metabolites as well as those of multidrug regimens. The method would be particularly well suited for the study of new drugs and their potential metabolites. In future studies, the use of the patients' own tumor cells as the "target", when these are able to grow in culture, may be superior in the search for a correlation between pharmacodynamic and clinical outcome.

To illustrate the applicability of this new pharmacodynamic approach, we studied the PCA of plasma from acute myeloblastic leukemic patients receiving daunorubicin (DNR). We selected this population of patients because they were treated with DNR, a major antitumor agent with multiple plasma metabolites that appear after administration of the parent compound [11]. Daunorubicinol (DNRol), the principle metabolite of DNR, is also active [3, 11] and, in vitro, is retained in leukemic cells longer than is DNR [13]. In vivo, after administration of DNR, the plasma exposure to DNRol is much greater than is the exposure to the parent compound [11–13]. The extent to which the other metabolites of DNR possess antitumor activity, are synergistic with, or antagonize the antitumor activity of DNR is currently unknown.

Materials and methods

Patient characteristics. From December 1987 to October 1988, 11 patients with acute leukemia (10 acute myeloblastic leukemia and 1 chronic myelocytic leukemia in blast crisis) were studied prospectively (Table 1). After giving written, informed consent, each patient received induction therapy consisting of a 7-day continuous infusion of cytosine arabinoside (Ara-C), at a dosage of 200 mg m^{-2} day^{-1}, and 45 mg m^{-2} day^{-1} DNR, as an i. v. bolus injection on days 1, 2 and 3. Three patients, who were more than 60 years of age, received only 30 mg m^{-2} day^{-1} DNR. Patients ranged in age from 32 to 77 years. Four were men and 7 were women. Of the 11 patients, 9 were undergoing their first induction chemotherapy and had never received any prior form of chemotherapy. None of the patients had liver dysfunction; 3 had renal insufficiency (Table 1). Five patients achieved a complete remission after therapy and 2 were non-responders. Four patients were not evaluable for response: 3 because of early death and 1 because of loss to follow-up (Table 1). All studies involving human volunteers were approved by the University of Maryland Human Volunteers Research Committee.

Patient sample acquisition. Immediately prior to any DNR treatment, 25 ml blood, for use in control studies, was collected aseptically into sterile, heparinized Vacutainer tubes (Becton Dickinson Inc., Rutherford, N. J.). Then, 10 ml of heparinized blood was obtained at 5, 10, 15, 20, 30, 60 min, 2, 6, 12 and 24 h after the end of the DNR i. v. bolus injection. If the patient was studied after the second or the third bolus administration

of DNR, blood was also collected just before that dose was given. Plasma samples were obtained by centrifugation at 400 *g* for 15 min. They were stored at –20° C in darkness for a maximum period of 9 months until they were assayed, on the same day, for PCA and concentrations of DNR and DNRol.

Materials. DNR was purchased as Cerubidine (Wyeth Laboratories Inc., New York, N. Y.). DNRol was produced enzymatically from DNR, using rat kidney homogenate, and purified by silicic acid column chromatography, as described by Bachur et al. [1, 2]. The final purity of DNRol was determined to be >95% by thin-layer chromatography using 250-μm Silica Gel G plates and a solvent system consisting of $CHCl_3$/methanol/acetic acid/water (80 : 20 : 14 : 6, by vol.) [4] and >99% when checked by the HPLC system described below.

Plasma DNR and DNRol determination. Plasma concentrations of DNR and DNRol were determined by HPLC with a modification of the technique reported by Erb et al. [8]. Plasma samples (1 ml) were mixed with 30 μl 1 μM doxorubicin internal standard (IS), extracted with 2 ml chloroform/isopropanol (1 : 1, v/v), and mixed with sufficient $(NH_4)_2SO_4$ to give a saturated solution [4]. After centrifugation at 31 000 *g* for 15 min, the supernatant phase was removed, dried under nitrogen, and reconstituted in 400 μl mobile phase. Each sample was filtered through a 0.45-μm filter (μ star LB, Costar Corporation, Cambridge, Mass.), and 200 μl aliquots were analyzed with a HPLC system consisting of a Waters model 510 pump (Waters Associates, Milford, Mass.), a C18 Alltech Econosil 10 μm (250 × 4.6 mm) column (Alltech Associates Inc., Deerfield, Ill.) and a RP C18 Newguard 7-μm (15 × 3.2 mm) guard column (Brownlee Labs, Santa Clara, Calif.). The mobile phase of acetonitrile/0.32 M *o*-phosphoric acid (27 : 73, v/v) was pumped at 1 ml/min. Fluorescence in the column eluant was detected with a Fluoromonitor detector (American Instrument Co., Silver Spring, Md.) fitted with a 470-nm excitation and a 585-nm emission cut-off filter. Concentrations of DNR and DNRol were quantified by comparing DNR/IS and DNRol/IS area-under-the-peak ratios to values from concomitantly performed standard curves. Standard curves were prepared in duplicate for each set of samples, using pooled human plasma to which known concentrations ranging from 0.01 μM to 1 μM DNR and DNRol were added. The limit of the sensitivity was 0.01 μM for DNR and DNRol. For concentrations of DNR between 0.05 μM and 0.5 μM, the day-to-day coefficients of variation for the ratio of area of DNR/IS were between 8.7% and 13.3% (n = 12). For DNRol, these coefficients of variation were between 9.4% and 9.6%. The intra-run means ± SD of the coefficients of variation, for the same range of concentrations, were between 6.2% ± 7.6 and 9.3% ± 8.4 for DNR; 9.9% ± 4.4 and 9.5% ± 7.7 for DNRol.

Cell culture. The HL-60 human promyelocytic leukemia cell line, used as the target cell system, was maintained in continuous suspension culture in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 μg/ml) and 10% (v/v) heat-inactivated fetal bovine serum, as previously described by our laboratory [15].

Flow-cytometric cell-survival assay. Previously, we had developed a sensitive flow-cytometric method for determining the number of cells surviving in suspension culture [16]. This method is a double-cyto-fluorescence assay, which utilizes fluorescein diacetate and propidium iodide. Viable cells are those that show a bright green fluorescence, induced by the intracellular metabolism of the diacetate to fluorescein, and a low red fluorescence of propidium iodide. In this assay, only intact viable cells are counted. Cells debris and dead cells are ignored. The number of viable cells per milliliter of culture-medium sample is determined by a timed count and knowledge of the flow-cytometer sample flow rate, as previously described [16]. The analyses were performed with a FACStar Plus flow cytometer (Becton Dickinson Immunocytometry Systems, Mountainview, Calif.).

PCA assay. To evaluate the in vitro cytotoxicity of plasma samples against the target cell line, 5×10^5 HL-60 cells, in logarithmic growth phase, were suspended in 1 ml of the same RPMI-1640 medium described above, except that the fetal bovine serum was replaced by non-heat-inactivated patient plasma (25%, v/v). Deoxycytidine (dCyd), at a final concentration of 500 μM, was added to counteract Ara-C activity in the plasma. The cultures were incubated for 72 h at 37° C in a 6% CO_2 atmosphere, after which time the number of viable cells remaining in each suspension was determined by flow cytometry, as previously described [16]. The coefficients of variation for the flow-cytometric measurement of the number of viable cells, in a single sample run twice, was below 10% when the number of cells was above 20 000, and below 15% for lower values. For duplicate or triplicate samples, the mean ± SD of the coefficients of variation for the PCA measurement was 6% ± 2.2. The numbers of viable cells/ml in each sample were expressed as a percentage of those in the drug-free pretreatment plasma control and called "cells surviving" in the equation below. The PCA of each sample was then calculated as the percentage of HL-60 cells affected by the drug:

PCA = cells affected (%) = 100–(cells surviving, %)

Mathematical modeling. Pharmacokinetic and pharmacodynamic modeling were accomplished by weighted, iterative, nonlinear, ordinary and maximum *a-posteriori* Bayesian [17] least-squares regression, using a computer program called ADAPT [7]. Weighting was by the inverse of the observation variance. Model discrimination was accomplished by Akaike's Information Criterion [19].

DNR and DNRol concentrations and PCA measured in plasma samples obtained following treatment were fitted simultaneously. DNR was modeled as having linear clearance, and two or three compartments of distribution, after bolus injection into the plasma compartment. Metabolism of DNR to DNRol was by a first-order clearance, with DNRol disposition described by a linear one- or two-compartment model. The PCA measured in these same samples (PCA_{obs}) was co-modeled with drug concentrations according to the following relationship:

$$\frac{PCA}{1\text{–}PCA} = \left[\frac{c_1}{IC_{50,1}} + \frac{c_2}{IC_{50,2}} + \frac{\beta \times c_1 \times c_2}{IC_{50,1} \times IC_{50,2}} \right]^h$$

where c_1 = DNR concentration after 4-fold dilution; c_2 = DNRol concentration after 4-fold dilution; $IC_{50,1}$ and $IC_{50,2}$ = Michaelis constants for DNR and DNRol, respectively (in this context, they represent the concentration of DNR or DNRol alone associated with a number of viable cells which is 50% of the control, after the 4-fold dilution); β = a coefficient of pharmacodynamic interaction (synergism, additivity or antagonism); and h = Hill's constant, which allows the surface to assume a spectrum of shapes. This formula is an adaptation of a standard two-drug pharmacodynamic interaction equation [6].

An in vitro "interaction panel", which characterized the pharmacodynamics of DNR and DNRol, alone and in combination, was developed for each patient as follows: fixed concentrations of DNR (0–1 μM) and/or DNRol (0–3 μM) were added to between 47 and 51 aliquots of the patient's pretreatment plasma. Then, the cytotoxic activity of each aliquot against HL-60 cells was determined using the methods described earlier. Because response, i.e. cytotoxic activity, is dependent on two variables (namely, the concentrations of DNR and DNRol), this interaction panel yields a standard surface instead of a standard curve. The standard surface developed for the interaction panel was fitted using the same pharmacodynamic equation for PCA as described above.

Based on the plasma concentrations of DNR and DNRol, determined by HPLC and adjusted for the 4-fold dilution used in the PCA assay, these standard surfaces were used to predict the PCA of the plasma samples drawn during DNR treatment.

The pharmacodynamic and pharmacokinetic data were co-modeled and integrated from 0 to 24 h. This process provided the areas under the PCA/time curves for the observed PCA (AUC_{obs}) and those predicted from the standard surface (AUC_{prd}). Concomitantly, we also computed the areas under the DNR and DNRol concentration/time curves (AUC_1, AUC_2). Areas were adjusted to a 45 mg m^{-2} dose of DNR (assuming a linear relationship between dose and AUC) and, for patients who were studied around the second or the third dose, to the first dose (using superposition).

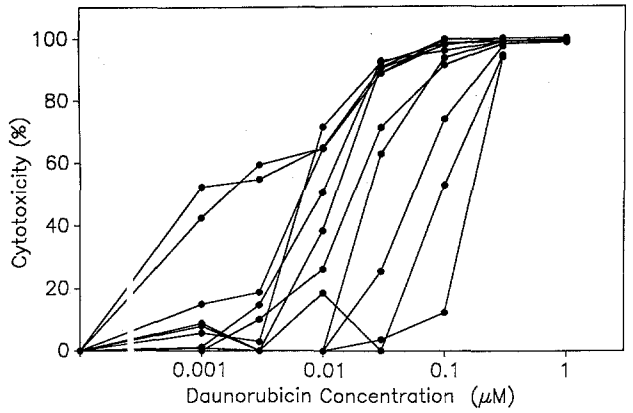

Fig. 1. Comparison of daunorubicin (DNR) standard curves in pretreatment plasma of different patients. Each curve corresponds to one patient, and represents the cytotoxicity of his or her pretreatment plasma against HL-60 cells after the addition of different concentrations of DNR. DNR concentrations are on a logarithmic scale

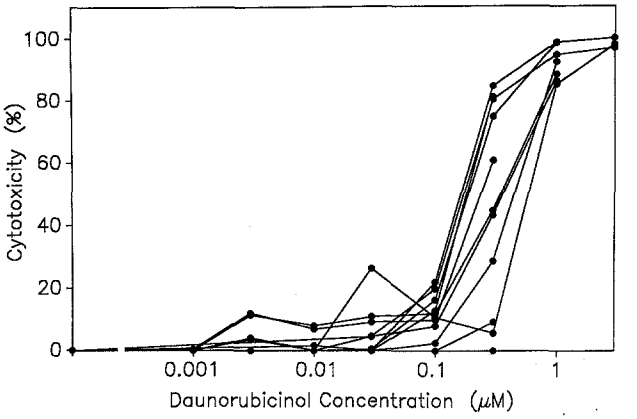

Fig. 2. Comparison of daunorubicinol (DNRol) standard curves in pretreatment plasma of different patients. Each curve corresponds to one patient, and represents the cytotoxicity of his or her pretreatment plasma against HL-60 cells after the addition of different concentrations of DNRol. DNRol concentrations are on a logarithmic scale

Statistical methods. Distributions of data that were markedly non-normal and/or had non-detectable "small" or non-identifiable "large" values were described using the median as a measure of central tendency and a range, defined as the median ± 1 quartile, as a measure of dispersion. Multiple linear regression was used to test the correlation between pharmacokinetic (AUC_1, AUC_2) and pharmacodynamic ($IC_{50,1}$, $IC_{50,2}$) parameters versus AUC_{obs}. Accordingly, each patient's AUC_1 was divided by his $IC_{50,1}$ and each patient's AUC_2 was divided by his $IC_{50,2}$. Multiple linear regression was performed with these two quotients and their product as independent variables and with AUC_{obs} as the dependent variable.

Results

Effect of pretreatment plasma on HL-60 cell growth

For all patient pretreatment plasma tested, we found that HL-60 cells survive and grow well in RPMI-1640 medium containing 25% (v/v) heparinized, non-heat-inactivated human plasma. However, the growth rate varied, depending on the patient plasma source. For the patients studied, the median number (± 1 quartile range) of viable HL-60 cells after 72 h of incubation was 1.00×10^6 [(7.89×10^5)–(1.56×10^6)] cells/ml. We also obtained similar variations in HL-60 growth rate when plasma from different normal volunteers, or different lots of fetal bovine serum were used (data not shown).

Cytotoxic activity of DNR or DNRol in pretreatment plasma

Figure 1 shows, for each patient's pretreatment plasma, the cytotoxic activity observed in response to concentrations of DNR alone. There was a substantial interpatient variability in the IC_{50} of DNR versus HL-60 cells. However, the reproducibility within an individual tested in duplicate was good with a coefficient of variation of 10.3% for the IC_{50}. Depending on the plasma source, the range of IC_{50} values approximated a 100-fold concentration difference. The median (± 1 quartile range) of IC_{50} for DNR was 0.012 μM (0.0068–0.056). In contrast, the sigmoidicity of each curve was not as variable among patients. The median Hill's constant (± 1 quartile range) was 1.9 (1.4–2.9). In the same pretreatment plasmas, DNRol was less active, but also less variable in its cytotoxic activity than DNR (Fig. 2). For DNRol, the median (± 1 quartile range) of IC_{50} was 0.33 μM (0.19–0.63), and of Hill's constant was 2.7 (2.2–3.7).

Observations similar to those above for DNR and DNRol were obtained when plasma from normal volunteers, or fetal bovine serum obtained from different pools, was used (data not shown). In contrast, minimal interexperiment variations were found for the cytotoxicity of DNR against HL-60 cells when RPMI-1640 medium containing 10% (v/v) fetal bovine serum, obtained from the same pool, was used [16]. This suggests that there was good reproducibility of our basic assay methods, and that the variability observed was due to interindividual differences in plasma.

Pharmacodynamic interactions between DNR and DNRol in pretreatment plasma

Figure 3 is a representative standard surface showing the cytotoxic activity (y axis) obtained with different combinations of concentrations of DNR (z axis) and DNRol (x axis) added to the pretreatment plasma of one of the patients (patient 11). Such pretreatment "interaction panels" were performed for each of the patients. Qualitatively, the shape of the standard surface given in Figure 3 shows that the majority of the PCA is due to DNR. In this case, a discernable contribution of DNRol was seen at concentrations greater than 0.03 μM. For each patient, we defined the parameters characterizing these standard surfaces by fitting the model, via the equation given in Materials and methods to the cytotoxic activity data. Table 2 gives the parameter values and the median (± 1 quartile range) of

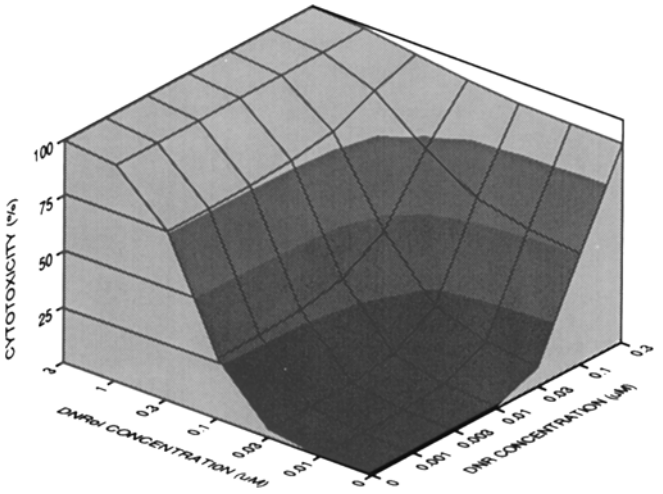

Fig. 3. Typical example of standard surface generated by the addition of different concentrations of DNR and/or DNRol to pretreatment plasma of patient 11, as described in Materials and methods. Each intersection of lines represents the cytotoxic activity of the pretreatment plasma containing the corresponding concentrations of DNR (z axis) and DNRol (x axis) against HL-60 cells in suspension culture. DNR and DNRol concentrations are on a logarithmic scale. The cytotoxic activity (y axis) is expressed as a percentage of cells affected compared to control (HL-60 cell suspension culture in medium containing same volume of pretreatment plasma)

Table 2. Parameter values characterizing the standard surface of each patient[a]

Patient	$IC_{50,1}$[b]	$IC_{50,2}$[b]	β[b]	h[b]	r^2[b]
1	0.014	0.25	0	2.6	0.971
2	0.072	f[c]	f[c]	3.1	0.982
3	0.019	0.35	0	1.7	0.980
4	0.028	0.65	0	3.1	0.958
5	0.0094	0.43	0	1.7	0.982
6	0.0035	0.29	0.59	1.0	0.927
7	0.0022	0.11	4.2	0.83	0.910
8	0.10	0.89	0.23	1.6	0.905
9	0.0074	0.18	0	2.1	0.982
10	0.011	0.17	0	1.8	0.978
11	0.10	0.17	0	1.9	0.908
Median ±1 quartile	0.014 (0.0074–0.072)	0.29 (0.17–0.65)	0 (0–0.59)	1.8 (1.6–2.6)	0.971 (0.91–0.982)

[a] Defined by fitting the model given in Materials and methods to the data

[b] $IC_{50,1}$ and $IC_{50,2}$ (μM), the Michaelis constants for daunorubicin (DNR) and daunorubicinol (DNRol), respectively (in this context, the concentration of DNR or DNRol alone associated with 50% cytotoxicity compared to control); β, a coefficient of pharmacodynamic interaction; h, Hill's constant, which dictates the degree of sigmoidicity; r^2, coefficient of determination

[c] Not identifiable

Table 3. Effects of dCyd on Ara-C cytotoxicity against HL-60 cells

[Ara-C] (μM)	Cells surviving[a] (%) (mean ± SD)[b]	
	Ara-C alone	Ara-C + 500 μM dCyd
0	100 ±2.5	100 ±1.0
0.1	63.9±1.0	98.9±5.9
0.3	43.3±7.0	101,2±6.0
1	8.5±0.2	104.8±0.6

[a] Percentage of cells surviving, number of viable HL-60 cells/ml RPMI-1640/plasma (75:25, v/v) expressed as a percentage of the number of viable cells in the respective controls. The mean ± SD numbers of viable cells in the Ara-C alone control was 1 095 844±27 041 viable cells/ml and 1 295 729±13 060 in the dCyd alone control.

[b] Means ± SD refer to triplicate samples with plasma cytotoxic activity (PCA) estimated twice on each of the samples

the IC_{50} of DNR ($IC_{50,1}$), IC_{50} of DNRol ($IC_{50,2}$), β and h, which defined the standard surfaces of the 11 patients. The mathematical model fitted the data very well; the range of r^2 was between 0.905 and 0.982 (Table 2). There was no trend to bias. The median values for $IC_{50,1}$ and $IC_{50,2}$ derived from the entire interaction panel (Table 2) were very similar to those obtained by fitting the data for DNR and DNRol alone. The median value of the pharmacodynamic interaction coefficient, β, was 0 with a ±1 quartile range of 0–0.59. These data ($0 < \beta < 1$) are consistent with an additive interaction between DNR and its metabolite DNRol. As such, additivity was seen in all but one (patient 7) of the patients.

Abrogation of Ara-C effects by dCyd

We found that the concentration of dCyd (500 μM) used is sufficient to thwart the cytotoxicity of Ara-C, at final concentrations up to at least 1 μM, against HL-60 cells in RPMI-1640/human plasma (75:25, v/v) (Table 3). This would correspond to an Ara-C plasma concentration of 4 μM prior to the dilution that is made in the PCA assay. As reported by others [5, 10, 18], this concentration of Ara-C is greater than the highest concentration observed in patients treated with the regimen used in this study. As illustrated in Figure 4, we also observed that 500 μM dCyd±0.1 or 1 μM Ara-C does not affect the cytotoxic activity of DNR in medium containing 25% human plasma.

Integration of pharmacokinetics and pharmacodynamics

For each patient, 24-h profiles were obtained by simultaneously fitting the linked pharmacokinetic/pharmacodynamic model, described in Materials and methods, to the observed concentrations of DNR and DNRol as well as to the corresponding PCA_{obs}. The cytotoxic activity expected in each plasma sample was also predicted from the fitted standard surfaces (see above) as a function of the assayed concentrations of DNR and DNRol within that sample. As an example, the DNR and DNRol plasma concentrations and the corresponding PCA_{obs} and PCA_{prd} profiles for patient 11 are graphically depicted in Fig. 5. In this figure, PCA_{obs} and PCA_{prd} are those corresponding to a 4-fold dilution of the plasma that is inherent in the determination of PCA. The plasma concentration of DNR peaked at the

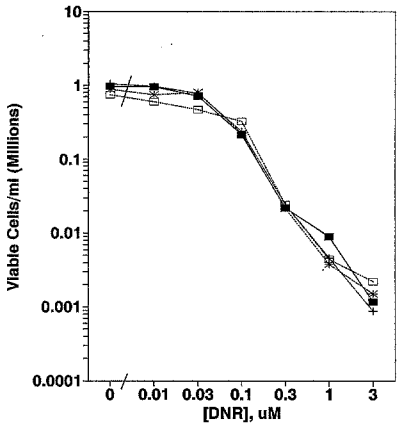

Fig. 4. Effect of dCyd ± Ara-C on DNR cytotoxic activity on HL-60 cells. Cells, in suspension culture (RPMI-1640/human plasma, 75:25, v/v), were exposed to the indicated concentrations of DNR ± dCyd and Ara-C for 72 h, after which the numbers of viable cells/ml medium were measured by the flow-cytometric cell-survival assay described in Materials and methods. ■, Control; +, 500 μM dCyd; – – ✳ – –, dCyd+0.1 μM Ara-C; □, dCyd+1 μM Ara-C

Table 4. Areas under the DNR and DNRol concentration curves (AUC_1 and AUC_2) and under the PCA_{obs} and PCA_{prd} (AUC_{obs}, AUC_{prd}) curves versus time (0–24 h)[a]

Patient	Pharmacokinetics		Pharmacodynamics		
	AUC_1 (μmol l^{-1} h)	AUC_2 (μmol l^{-1} h)	AUC_{obs} (% h)[b]	AUC_{prd} (% h)[b]	DIF (%)
8	0.56	2.1	1336	51	2509
2	0.056	6.2	447	59	658
4	0.33	1.8	215	38	469
11	1.10	4.6	1459	348	319
5	0.98	1.9	816	460	78
3	0.52	2.6	364	356	2
6	0.47	1.4	517	906	–43
9	0.32	1.5	240	438	–45
1	0.57	1.5	116	220	–47
7	0.94	3.8	823	1929	–57
10	0.69	1.6	228	551	–59

[a] Areas are adjusted to a 45 mg/m^2 and first dose of DNR. DIF reflects the percentage difference between AUC_{obs} and AUC_{prd} using the formula described in Results

[b] Percentage cells affected × h

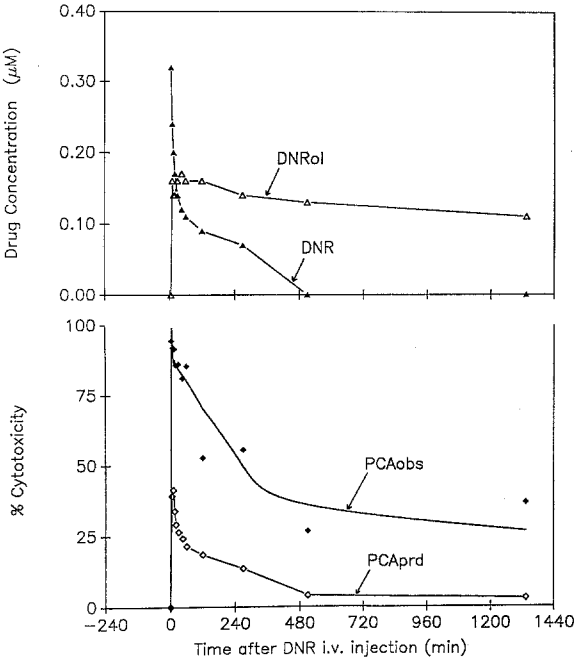

Fig. 5. Example of the profiles of DNR and DNRol plasma concentrations (*upper graph*), the corresponding observed plasma cytotoxic activity (PCA_{obs}), and the predicted PCA (PCA_{prd}) incorporating the 1:4 dilution of the plasma from the "interaction panel" (*lower graph*) during 24 h after DNR administration in patient 11. Drug concentrations were assayed by HPLC as described in Materials and methods

end of the i.v. bolus injection and then, in 9 of the 11 patients, declined rapidly. For all patients, the 24-h DNR concentration was very low, and often non-detectable. DNRol was generated quickly and was already measurable in plasma samples obtained 5 min after the end of the DNR injection. As previously described [11, 12, 14], plasma concentrations of DNRol exceeded those of DNR in most of the samples collected during the 24 h after DNR administration. At 24 h, a significant amount of DNRol was still present in the plasma of all of the patients. In the case shown (Fig. 5), PCA_{obs} paralleled DNR disappearance until 480 min, after which there was a plateau through 1360 min. The PCA_{prd} curve was, for the most part, dependent on the concentration of DNR. However, in this example (Fig. 5), the PCA_{obs} was consistently and markedly greater than PCA_{prd} by the standard surface.

Within each patient, the model was able to fit the pharmacokinetic and pharmacodynamic data very well. Integration of DNR and DNRol plasma concentrations, from 0 to 24 h after DNR administration, provided the AUC_1 and AUC_2, respectively. Integration of the PCA_{obs} and PCA_{prd} values, over the same period of time, provided, the areas under the cytotoxic activity versus time curves (0–24 h), AUC_{obs} and AUC_{prd} respectively. In Table 4, the AUC_1, AUC_2, AUC_{obs} and AUC_{prd} (all normalized to 45 mg m^{-2} and first dose) are shown for each patient. The AUC_1 showed a 19.6-fold interpatient variation, and the AUC_2 showed a 4.4-fold variation. The ratios of AUC_2 to AUC_1, as taken from Table 4, were also markedly different among patients, with a range from 1.9 to 111. For PCA, the AUC_{obs} also varied substantially between patients. There was a 12.6-fold variation in the AUC_{obs} among these patients. As described in the statistical methods, multiple linear regression was used to determine what proportion of this variance in AUC_{obs} was associated with variance in pharmacokinetics and in vitro cytotoxicity of DNR and DNRol. None of the independent variables was significantly related to AUC_{obs} (at $P < 0.05$). The percentage of variance in AUC_{obs} explained by the regression was 23. Clearly, most of the interpatient variance in integrated PCA was due to factors other than variance in pharmacokinetics or variance in factors present in pretreatment plasma.

Comparison of the observed versus predicted pharmacodynamics

To compare the PCA_{obs} in the plasma after DNR treatment with the cytotoxicity that we predicted, we calculated the percentage difference between the AUC_{obs} and the AUC_{prd} by the formula:

$$\text{difference (\%)} = 100 \times \frac{(AUC_{obs} - AUC_{prd})}{AUC_{prd}}$$

This formula will give a percentage difference of 0 if $AUC_{obs} = AUC_{prd}$, >0 if $AUC_{obs} > AUC_{prd}$ and <0 if $AUC_{obs} < AUC_{prd}$. Table 4 gives the data for all the patients in order of decreasing percentage difference. Only one patient (patient 3) was well predicted by this assumption with a difference of 2%. Five patients (8, 2, 4, 11 and 5) showed a significantly higher cytotoxic activity in their plasma after DNR treatment than was predicted, and 5 patients (6, 9, 1, 7 and 10) had a significantly lower AUC_{obs} than that predicted.

Discussion

We have developed a new pharmacokinetic/pharmacodynamic model, using PCA. As an example, this model was employed to study the cytotoxicity and the behavior of DNR in acute myelocytic leukemic patients and to characterize interindividual differences in their pharmacodynamics. The model concomitantly fit the pharmacokinetic and pharmacodynamic data very well. The methods used (flow-cytometric cell-survival assay, analytical chemical assays, modeling) are easy to perform, demonstrating that this approach is technically feasible.

We showed that HL-60 cells are able to grow well in medium containing 25% non-heat-inactivated human plasma instead of fetal bovine serum. However, the growth rates of HL-60 cells were dependent on the plasma source (patient or normal volunteer). This is not surprising since, in our laboratory's experience, the growth rates of HL-60 cells cultured in fetal bovine serum also vary substantially among different lots of serum. It seems that there are factors, variable among individuals, contained or lacking in plasma that affect HL-60 growth rate. If further work is to be done with other cell lines or the tumor cells of a patient, the ability of these cells to survive in the appropriate plasma supplement must also be demonstrated.

As previously shown in other cell lines [3, 11], DNRol, like DNR, is cytotoxic to HL-60 cells in suspension culture. However, DNRol is much less potent than is DNR, as can be seen from the IC_{50} data shown in Table 2. Surprisingly, the toxicity of DNR to HL-60 cells was also very dependent on the human plasma into which the drug was added. There was a 50-fold variation in the IC_{50} of DNR among the patients studied. There was no correlation between DNR IC_{50} and the growth rates of HL-60 cells in these patients' pretreatment plasma. In other words, a low DNR IC_{50} did not systematically correspond to either a fast or slow growth rate in pretreatment plasma. Thus, significant variability exists among patients in the ability of DNR to exert antitumor activity in their plasma, even before the drug is metabolized. Possible explanations for this variability are interpatient differences in binding between DNR and plasma constituents, or the presence of DNR-inhibiting or -stimulating substances in the plasma of some patients. The fact that DNR cytotoxicity against HL-60 cells in drug-free plasma from normal volunteers was similarly variable suggests that the variation observed in patients' pretreatment plasma was not specifically related to their acute leukemia or to other drugs (e.g. antimicrobials, antiemetics) that they were receiving. There also was no relationship between this variability among patients and their blood chemical parameters (protein concentration, renal or hepatic functions) or their other medications. In contrast to DNR, DNRol displayed less variation in IC_{50} based on pretreatment plasma.

PCA, expressed as AUC_{obs} measured after a fixed dose of DNR, was also very variable (12.6-fold) among patients. Therefore, the total in vitro cytotoxic activity against HL-60 cells that patients generate in their plasma after a given dose of DNR differs widely from patient to patient. As have other authors, we noted very large interindividual differences in the pharmacokinetics of DNR and DNRol [12, 13]. But according to the multiple linear regression analysis performed, the substantial interpatient variability in AUC_{obs} was not correlated with interpatient differences in DNR and DNRol pharmacokinetics, in vitro growth rates and/or in vitro drug activity (e.g., IC_{50} values) in pretreatment plasma.

In an effort to explore the factors that cause the intersubject variance in pharmacodynamics, the AUC_{obs} was compared to AUC_{prd}. If we assume that the patient-specific factors and binding present in the pretreatment plasma that affect cell growth do not change during therapy, then the large differences between AUC_{obs} and AUC_{prd}, seen in the subset of 4 patients, may be due to unidentified active metabolite(s) of DNR. If we discard the latter assumption, then the differences in AUC_{obs} and AUC_{prd} may *also* be attributed to cytotoxic or stimulating factors produced in the plasma by the host after treatment. Because PCA was measured for each patient against the same cell line (HL-60 cells), this interpatient variability raises the possibility that treatment failure may not be due solely to resistance at the level of the neoplastic cell. These findings support the hypothesis that interpatient variability in metabolism to active species may contribute to the likelihood of response. The possibility that another active metabolite and/or host-specific factors might be the cause of the large differences in observed versus predicted PCA is a hypothesis worthy of further evaluation. Benjamin et al. have already described interindividual variability in the production of an aglycone metabolite of doxorubicin [4], but there are no data about the activity of this metabolite.

Since only 7 of these patients were evaluable for response (see Table 1), no conclusions about the relationship between the measurement of the PCA generated after DNR treatment and clinical outcome are possible at this time. To identify such relationships will require larger series that also consider simultaneously other variables that are known to be associated with prognosis, such as other antineoplastic drugs used, age, or cytogenetics. Ideally, such studies could utilize the patient's own tumor cells, as "tar-

get", provided that those cells uniformly grow well in vitro in that patient's own pretreatment plasma.

The differences among patients in their AUC_{obs}, or between the observed and predicted data, are not likely to be due to a method-related variability but, rather to patient heterogeneity. We found good reproducibility in repeated studies. The data were also internally consistent; for all the patients, the pharmacokinetic/pharmacodynamic model fitted the data very well. To minimize error, the DNR and DNRol plasma concentrations, the PCA_{obs} and the interaction panel for each patient were determined on the same day. The majority of the work was performed within 2 weeks to assure uniformity of instrument calibration and HL-60 cell growth.

PCA measurement, in theory, is a potentially useful approach to the pharmacodynamic study of anticancer drugs. PCA reflects many factors other than measurable drug concentration and may be useful, in the future, to the indirect study of the metabolism of antineoplastic compounds as well as in developing pharmacodynamic relationships between drug administration and clinical outcome.

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