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COMPARATIVE CYTOTOXICITY, DNA SYNTHESIS INHIBITION AND DRUG INCORPORATION OF EIGHT ANTHRACYCLINES IN A MODEL OF DOXORUBICIN-SENSITIVE AND -RESISTANT RAT GLIOBLASTOMA CELLS

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Abstract—We have compared the growth inhibition, DNA synthesis inhibition and cell incorporation of eight anthracyclines in a model of doxorubicin-sensitive and -resistant rat C6 glioblastoma cells. The anthracyclines studied were both the reference molecules (daunorubicin, doxorubicin) and the new drugs recently introduced in clinical use or in trials (epirubicin, idarubicin, pirarubicin, esorubicin, rubidazone, 4′-deoxy-4′-iododoxorubicin). We have shown that the *in vitro* growth inhibition was correlated with the LD_{50}. The new anthracyclines were more potent than the reference drugs in the sensitive cells and the resistance patterns revealed a reduced cross resistance of idarubicin, pirarubicin and 4′-deoxy-4′-iododoxorubicin towards the doxorubicin-resistant line. DNA synthesis inhibition occurred for much higher doses than growth inhibition in sensitive cells, but for similar doses in resistant cells. This suggests that different mechanisms could be involved in the mechanism of growth inhibition in sensitive and resistant cells. For similar exposures, reduction of drug incorporation was a general feature in the resistant line when compared to the sensitive one. However, no correlation was shown, for the various drugs, between the reduction of incorporation and the resistance factor. Moreover, the intracellular concentration required for growth inhibition is much higher in resistant cells than in sensitive cells, suggesting that increased drug efflux might not be the only mechanism to explain drug resistance.

Several new anthracyclines have recently entered the phase II and III clinical studies in recent years. Whereas several hundreds of compounds have been synthesized, especially in the laboratory of Arcamone [1], only few of them have presented a clinical interest. Preclinical studies of the anthracyclines which are presently in clinical trials have been made on several independent tumor systems. At the cellular level, different kinds of cells in culture have been used to characterize the pharmacologic parameters of the drugs such as uptake, growth inhibition or metabolism. Several reviews have compared the various preclinical models for anthracycline evaluation and selection [2, 3].

We have developed a comparative study of eight of the anthracyclines presently used in clinics, either routinely or in trials, on the same model of tumor cells. We have chosen a rat glioblastoma, C6, already studied in our laboratory, which has been extensively studied for many years. Its doxorubicin resistant counterpart was selected in our laboratory [4]; the pharmacologic parameters of doxorubicin uptake and efflux, as well as its effect on DNA synthesis have already been characterized.

The anthracyclines studied were daunorubicin, the first anthracycline isolated, active on leukemia [5]; doxorubicin, or adriamycin, widely used in the treatment of hematologic malignancies and solid tumors [6]; zorubicin or rubidazone, a semi-synthetic derivative of daunorubicin, used in France in the remission induction therapy of acute leukemia [7]; epirubicin (4′-epidoxorubicin), which has been shown to be equipotent and less cardiotoxic than adriamycin [8] and which has received a commercial agreement in several Western countries; idarubicin (4-demethoxy-daunorubicin), which is lipophilic enough for oral administration, presently in phase II and III studies [9]; pirarubicin (4′-tetrahydropyranyldoxorubicin), which is presently in phase II studies, having been selected for lower cardiac toxicity in animal models [10]; esorubicin (4′-deoxydoxorubicin), which has been recently withdrawn from phase II clinical trials [11]; and 4′-deoxy-4′-iododoxorubicin, which recently entered a phase I trial in Italy and France [12]. This panel of anthracyclines contains thus the most presently relevant molecules of this family of antitumor antibiotics.

Several cellular pharmacologic parameters are of importance to the physician for the designing of therapeutic protocols for clinical trials; they are often used in parallel for the evaluation of maximal tolerated dose during the phase I study. The variability of the preclinical models used for each molecule prompted us to evaluate the cellular pharmacology of all these molecules in a single model. The most relevant parameters are the respective sensitivity of the wild cells to the various molecules, or, for the doxorubicin-resistant cells, the cross-resistance between the new anthracyclines and the reference molecule, doxorubicin. Other points of interest are the possible links between drug accumulation and growth inhibition and between DNA synthesis inhibition and growth inhibition.

MATERIALS AND METHODS

Materials. The different drugs were generous gifts

from Laboratoire Roger Bellon (doxorubicin, daunorubicin, rubidazone, pirarubicin) and from laboratories Farmitalia-Carlo-Erba (epirubicin, esorubicin, idarubicin, 4′-iododoxorubicin). All of them were clinical formulations, except 4′-iododoxorubicin which was a pure chemical product.

The drugs were dissolved in sterile bidistilled water (at concentrations of 1–10 mg/ml depending on the drug) and stored at −20°.

Media and sera for cell cultures originated from Seromed, Petri dishes from Nunc. [^{3}H-methyl]-thymidine and [^{14}C] doxorubicin were purchased from Amersham (France). Liquid scintillation medium was provided by Beckman.

Cell cultures. The C6 clone originated from a brain tumor induced in rats by *N*-nitrosomethylurea [13]. The C6 0.5 E clone was derived from the above clone in our laboratory by exposure to stepwise increasing amounts of doxorubicin, and selected for its stability to grow in the presence of 0.5 μg/ml of doxorubicin in the culture medium [4]. The cells were cultivated as monolayers in Petri dishes with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotic mixture at 37°, in a humidified atmosphere containing 5% CO_2. The cultures were replicated each week and the medium was changed each two or three days, depending on the cell density.

Growth curves of the two cell lines were ascertained in order to carefully establish reproducible experimental conditions. We have determined that the doubling times of the cell populations were 18 ± 2 hr and 36 ± 2 hr for the C6 and the C6 0.5 E lines respectively. We noted that the C6 0.5 E line presented a density-dependent growth inhibition more important than that presented by the C6 wild strain. On the basis of these results, we have determined the number of cells to be seeded in Petri dishes and the optimal size of those Petri-dishes, in order to obtain for the experiments an identical number of sensitive or resistant cells in an exponential state of growth and in a sufficient amount for accurate determinations.

Evaluation of growth inhibition. 4×10^4 C6 control cells were seeded in 10 cm^2 Petri dishes with 3 ml of medium, and 25×10^4 C6 0.5 E cells were seeded in 20 cm^2 Petri dishes with 5 ml of medium without drug. Three days later, when the number of cells reached approximately 5×10^5 cells per dish for both lines, the medium was substituted by 3 ml new medium containing various concentrations of drug (0.0032–100 μg/ml) and incubation was performed for 2 hr at 37°. After this drug exposure, monolayers were washed twice with sterile 0.15 M NaCl, replaced by normal medium and reincubated for 48 hr and 96 hr for the C6 and the C6 0.5 E cells respectively; these times corresponded approximately to two cell cycles for each cell line. For the estimation of cell survival, monolayers were washed twice with 0.15 M NaCl and suspended in culture medium with the help of a trypsin solution. Cells were counted in an automatic hemocytometer (Royco-Cell Crit 920 A).

All measurements were performed in triplicate and three independent experiments were performed. The cytotoxicity was expressed as GIC_{50}, i.e. the concentration causing 50% growth inhibition as compared to controls incubated simultaneously in the absence of drug.

Drug incorporation and [^{3}H-methyl] thymidine incorporation. For these evaluations, 8×10^4 C6 cells were seeded in 10 cm^2 Petri dishes with 3 ml medium, or 50×10^4 C6 0.5 E cells were seeded in 20 cm^2 Petri dishes with 5 ml of medium without drug. The medium was changed three days later; on the fourth day, the number of cells reached approximately 2×10^6 cells/dish. Drug incorporation was measured by substituting the medium by 3 ml new medium containing various concentrations of drug (0.032–320 μg/ml) and the dishes were incubated at 37° for 2 hr. One hour before the end of the drug exposure, 1 μCi of [^{3}H-methyl]thymidine per dish was added. Then, the monolayers were washed twice with 0.15 M NaCl, harvested after gentle stirring and pelleted at 3000 rpm for 5 min. These steps were rapidly performed in order to avoid any efflux of the drug; 0.5 ml of bidistilled water and 0.5 ml of 40% trichloroacetic acid were successively added and the samples were kept at 4° overnight, then centrifuged during 30 min at 3000 rpm. The acid-soluble part was used to evaluate the intracellular concentration of the non-covalently bound drug by fluorometry (Jobin-Yvon NE1 spectrofluorometer) with excitation and emission wavelengths set at the maximum fluorescence of each drug in the trichloroacetic solution (around 470 nm for excitation and 550 nm for emission). The acid-insoluble pellet was solubilized with 1 M NaOH and used to evaluate both the protein content [14] and the [^{3}H] radioactivity in a Beckmann L 1210 liquid scintillation spectrometer. We measured with [^{14}C] labeled doxorubicin the amount of drug that remained in the acid-insoluble pellets; it represented 10–12% of the total radioactivity incorporated in sensitive cells and 20–40% of the total radioactivity incorporated in resistant cells.

All incubations were performed in triplicate and three independent experiments were performed. In all cases, the incorporation of [^{3}H-methyl]thymidine was referred to controls realized in the same conditions and incubated without drug.

It was possible to define a TIC_{50} value, i.e. the concentration of drug providing a 50% decrease of [^{3}H]thymidine incorporation.

RESULTS

1. *Cytotoxicity in wild cells*

Table 1 presents the comparative growth incorporation of the eight anthracyclines studied in C6 sensitive cells. The values of GIC_{50} range from 0.010 μg/ml for idarubicin to 0.175 μg/ml for rubidazone. Those values can be compared to the LD_{50} of the drugs. It clearly appears that these parameters are closely related and give almost the same classification of the eight drugs.

2. *Cytotoxicity in doxorubicin resistant cells*

We show in Table 1 that the cross-resistance of the cells towards the eight drugs is highly variable. The degree of resistance was evaluated by the ratio of the GIC_{50} obtained in resistant and sensitive cells. The resistance of our C6 0.5 E line to doxorubicin

Table 1. Growth inhibition and DNA synthesis inhibition of C6 and C6 0.5 E cells by the various anthracyclines

Drug	LD_{50} in mice (mg/kg)	C6 cells GIC_{50} (μg/ml)	C6 cells TIC_{50} (μg/ml)	C6 0.5 E cells GIC (μg/ml)	C6 0.5 E cells TIC_{50} (μg/ml)	Resistance factor
Idarubicin	4.9	0.010 ± 0.005	0.33 ± 0.19	0.78 ± 0.43	1.43 ± 0.12	78
Iododoxorubicin	9.7	0.012 ± 0.005	0.93 ± 0.25	0.81 ± 0.34	1.90 ± 0.57	67
Esorubicin	14.1	0.014 ± 0.006	0.34 ± 0.14	11.8 ± 4.5	5.00 ± 0.85	816
Pirarubicin	14	0.025 ± 0.014	0.26 ± 0.10	3.58 ± 0.98	0.75 ± 0.16	143
Daunorubicin	24.9	0.028 ± 0.003	0.53 ± 0.08	21.7 ± 7.0	12.3 ± 1.5	779
Doxorubicin	21.9	0.076 ± 0.020	1.15 ± 0.50	30.3 ± 5.7	33.5 ± 2.1	398
Epirubicin	29.3	0.123 ± 0.050	1.37 ± 0.18	>1000	>320	>8,000
Rubidazone	35	0.175 ± 0.078	2.15 ± 0.49	>320	25.0 ± 9.9	>2,000

GIC_{50} are evaluated as the drug extracellular concentrations providing a decrease of 50% of cell survival. 5×10^5 cells were incubated for 2 hr in the presence of various concentrations of the drugs; cell survival was evaluated two cell cycles later by cell counting in an hemocytometer. Ratio of GIC_{50} in resistant and sensitive cells is the resistance factor.

TIC_{50} are evaluated as the drug extracellular concentrations providing an inhibition of 50% of [^{3}H-methyl] thymidine incorporation. 2×10^6 cells were incubated for 2 hr in the presence of various concentrations of the drugs. [^{3}H-methyl] thymidine was added during the second hour of incubation.

Table 2. Intracellular incorporation of the various anthracyclines after exposures to the same drug incorporation (1 μg/ml) or to concentrations equal to the GIC_{50} or the TIC_{50} of the drug

	Exposure to 1 μg/ml (μg/mg prot)		Exposure to GIC_{50} (μg/mg prot)		Exposure to TIC_{50} (μg/mg prot)	
	C6 cells	C6 0.5 E	C6 cells	C6 0.5 E	C6 cells	C6 0.5 E
Idarubicin	5.00 ± 1.39	3.05 ± 1.26	0.070	2.8	1.8	5.2
Iodoxorubicin	3.85 ± 1.04	3.07 ± 1.30	0.045	2.2	3.4	6.0
Esorubicin	5.26 ± 0.39	0.58 ± 0.15	0.085	11	1.9	3.3
Pirarubicin	5.23 ± 0.58	1.12 ± 0.41	0.18	13	1.6	0.72
Daunorubicin	4.08 ± 0.98	0.24 ± 0.10	0.060	25	1.8	4.6
Doxorubicin	1.64 ± 0.44	0.15 ± 0.01	0.21	1.3	1.7	1.9
Epirubicin	2.19 ± 0.19	0.20	0.24	>40	3.0	>40
Rubidazone	1.30 ± 0.37	0.13 ± 0.01	0.30	>30	2.3	2.3

2×10^6 cells were incubated for 2 hr in the presence of various concentrations of drugs. Net incorporation of drugs was estimated by fluorometry with excitation and emission wavelengths set at the maximum fluorescence of each drug in the trichloroacetic solution. Results obtained for exposure at 1 μg/ml are presented in the first two columns; incorporation at GIC_{50} and TCI_{50} exposure doses were evaluated by interpolation between two actual drug concentrations to which the incorporation of drugs by the cells were determined.

was about 400. These cells exhibited a low cross-resistance to idarubicin, 4′-iodo-4′-deoxydoxorubicin and pirarubicin, whereas they displayed a very high resistance to epirubicin and rubidazone.

3. *Inhibition of DNA synthesis*

The inhibition of [^{3}H]thymidine incorporation in our cells is presented in Table 1 as IC_{50} values for this parameter. It appears that there is no exact correlation between the GIC_{50} and the TIC_{50} values neither in sensitive nor in doxorubicin-resistant cells. In sensitive cells, the ratio of the IC_{50} obtained for the two parameters ranges from 10 (pirarubicin) to 77 (iododoxorubicin). In contrast, this ratio ranges from 0.08 (rubidazone) to 2.3 (iodododoxorubicin) in resistant cells.

4. *Incorporation of anthracyclines*

Table 2 presents the incorporation of each anthracycline in C6 sensitive and doxorubicin-resistant cells. We have selected for presentation only one drug concentration (1 μg/ml). Only the TCA-extractible drug was measured in this study. Sensitive cells incorporated more drug than resistant ones, the difference being slight for some drugs such as idarubicin of 4′-iodo-4′-deoxydoxorubicin and very important for other drugs such as epirubicin or daunorubicin. In both sensitive and doxorubicin-resistant cells, high levels of incorporation were correlated with important growth inhibition and DNA synthesis inhibition, and conversely.

We have evaluated the incorporation of drug in cells exposed to a concentration equal to the GIC_{50} or TIC_{50} as defined before (Table 2). We observed that drug incorporation in cells exposed to the TIC_{50} are not very different from a drug to another one in sensitive cells. However, the incorporation of a drug in resistant cells exposed to the GIC_{50} or to the TIC_{50} varied greatly from one drug to another one; these differences in incorporation at GIC_{50} and TIC_{50} reflect in fact the differences between GIC_{50} and TIC_{50} of the drugs. It is worth noting that, for a similar inhibition of growth (50%), the resistant cells had incorporated 6–300 times more drug than the sensitive ones; and

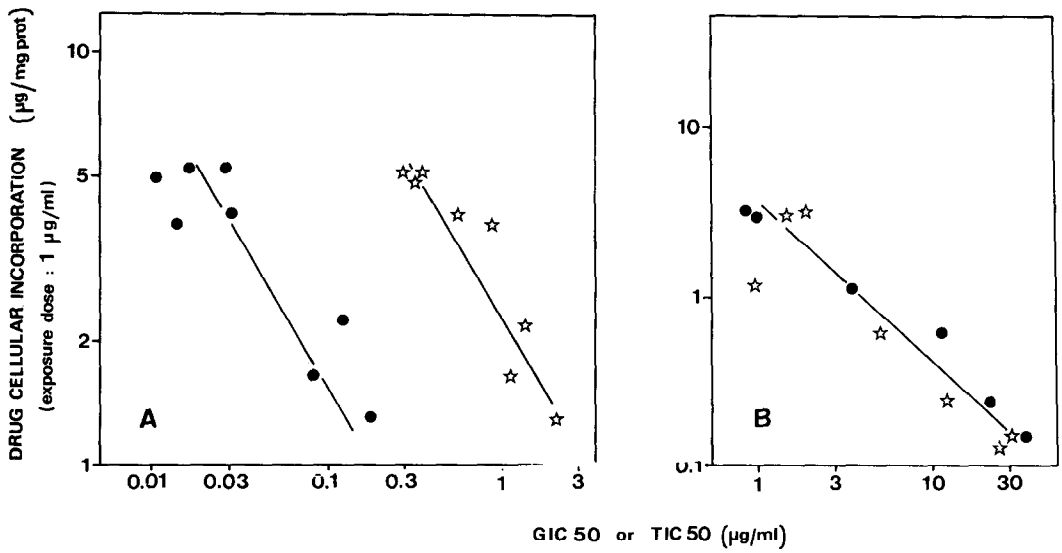

Fig. 1. Relationship between drug cellular incorporation for the same exposure dose (1 µg/ml), and GIC_{50} (●) or TIC_{50} (☆) in C6 sensitive (A) and doxorubicin-resistant (B) cells. GIC_{50} was defined as the dose responsible for a 50% inhibition of growth, and TIC_{50} as the dose responsible for a 50% inhibition of thymidine incorporation into DNA. Drug incorporations, and inhibitions growth and DNA synthesis were evaluated as described in the text and in the legends of Tables 1 and 2.

that, for a similar inhibition of DNA synthesis (50%), the resistant cells had incorporated similar amounts of drug as sensitive cells, excepted for one drug.

We have plotted on Fig. 1 the relationship between drug cellular incorporation for an extracellular concentration of 1 µg/ml and the GIC_{50} and TIC_{50}. A linear relationship is observed between the logarithm of GIC_{50} or TIC_{50} of a drug and its intracellular concentration. Two different curves are obtained for sensitive cells according to the parameter used (growth inhibition or DNA synthesis inhibition); in contrast, these curves are mixed up for resistant cells.

DISCUSSION

It appears from our results that the newer anthracyclines are more potent than the parent original molecules from which they derive, daunorubicin and doxorubicin. This is not surprising since these new drugs have been selected from *in vitro* screening models because of their increased potency. It is worth noting that the new molecules such as idarubicin, pirarubicin or 4′-deoxy-4′-iododoxorubicin are more lipophilic than the original ones or their first analogues. There is a highly significant correlation between the *in vitro* activity of the drugs and their *in vivo* toxicity, evaluated as LD_{50} in rodents. This strongly suggests that *in vitro* and *in vivo* toxicities share some common mechanism. Such a correlation would be probably obtained with the same set of drugs in various cellular models. It is worth noting that the recommended doses in clinics follow the same order as the *in vitro* GIC_{50}: for instance 10 mg/m^2 for idarubicin, 50 mg/m^2 for doxorubicin and 200 mg/m^2 for rubizadone, which are the recommended doses in humans, can be compared with the GIC_{50} in sensitive cells, which are respectively 0.010, 0.076 and 0.175.

It is now well established that resistance to doxorubicin is in fact a multidrug resistance which crosses with all the anthracyclines and with numerous unrelated drugs such as vinca alkaloids or actinomycin D [15]. It is generally admitted that this resistance is due to the increased activity of a membrane high molecular weight glycoprotein which pumps the various drugs out of the cells [16]. It has been widely observed but not explained that the degree of resistance of each *in vitro* cell line may vary greatly from one drug to the other, even within the same drug family. One of the goals of the development of new anticancer drugs is to obtain new molecules devoid of cross resistance with classical ones. It is, however, not known if the *in vitro* models for multidrug resistance may be valid for the study of clinical drug resistance. Therefore, it is not possible to assert that a reduced cross-resistance *in vitro* of a new drug corresponds to a broader *in vivo* spectrum or at least to an activity of this drug against acquired resistance to a classical drug. It must be pointed out that the degree of resistance of *in vitro* cell lines is very high (100–1000) whereas the resistance of human tumors may be only of 2–5 [17]. It would be expected, if the resistant models have some relevance with clinical resistance, that a reduced cross resistance between a new analogue and a classical drug would lead to the possible extension of the clinical use of the analogue. Our results show a reduced cross resistance of 4′-deoxy-4′-iododoxorubicin, idarubicin and pirarubicin towards our doxorubicin-resistant cell line; this is in agreement with previous observations made on other resistant models during the preclinical screening of these drugs [18–20].

The exact mechanism by which doxorubicin and

its analogues kill the cells is not known. It was thought that it occurred through an inhibition of DNA or RNA synthesis after drug intercalation in DNA [21]. Other hypotheses are inhibition of topoisomerase II [22], metabolic activation to reactive semiquinone free radical intermediates [23] or even membrane transduction of growth inhibition messages [24]. In sensitive cells, the concentrations of drug inhibiting by 50% the incorporation of [^{3}H]thymidine in DNA (TIC_{50}) are 10–80-fold higher than the concentrations of drug inhibiting by 50% the cell growth and survival (GIC_{50}). In contrast, in resistant cells, TIC_{50} and GIC_{50} are much closer, the TIC_{50} being either somewhat higher than GIC_{50} or somewhat lower, the only exception being rubidazone, for which the growth inhibition occurred for a >10 times higher dose than the inhibition of DNA synthesis. A possible explanation of these observations is that the growth inhibition is not related to DNA synthesis inhibition in sensitive cells, but could process through such an inhibition in the resistant cells. A similar conclusion was drawn by Charcosset *et al.* [25] from studies in another model of drug resistance. The lack of relationship between inhibition of DNA synthesis and cytotoxicity had been already emphasized by Siegfried *et al.* [26]. It is not possible to conclude from our results if interaction with topoisomerase II differs in the cell lines studied. Munger *et al.* [27] have recently studied intrinsically resistant hepatoma cells. They observed a close relationship between inhibitions of DNA synthesis and cell proliferation as in our selected doxorubicin-resistant cells.

Reduction of drug incorporation has often been considered as the primary cause for drug resistance. From the original works of Dano [28] or Skovsgaard [29] it is clear than an active efflux mechanism pumps the drugs out of the cells, and that the importance of this mechanism is quantitatively related to the degree of resistance. We have already shown in our cells that doxorubicin-resistance was accompanied by an active efflux of this drug out of the cells [4]. It was necessary to compare the various drugs of the anthracycline family in this respect. For most drugs, there is a linear relationship between reduction of incorporation and degree of resistance, as evident from the comparison of Tables 1 and 2. It is worth noting several exceptions to this rule, esorubicin, rubidazone and epirubicin being incorporated in doxorubicin-resistant cells to a higher extent than expected from cross-resistance values. It may be concluded that reduction of net drug incorporation is not the only determining factor in the expression of drug resistance. Similar conclusions were already drawn from other studies [30]. Our first measures were made at a fixed extracellular concentration (1 μg/ml) and for the same time (2 hr). We have calculated then the drug incorporation for an extracellular concentration as close as possible to the GIC_{50} and to the TIC_{50}. It clearly appears that reduction of drug incorporation cannot explain solely the resistance since much higher intracellular drug levels are required in resistant cells than in sensitive cells for the same growth inhibition. In contrast, DNA synthesis inhibition requires in most cases similar intracellular drug levels in sensitive and resistant cells. These results are in favor of the development, in resistant cells, of detoxication mechanisms supplementary to the reduced drug incorporation. Several explanations could account for these results: differences in intracellular drug metabolism or compartmentation may be responsible for this increase of drug tolerance in resistant cells; alterations of the ultimate targets of the drugs must also be considered; decreased ability to activate the drugs to reactive semiquinone-free radical species might also explain the phenomena observed, as well as the development of free radical detoxication mechanisms. This is presently under investigation.

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