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SHORT COMMUNICATIONS

Aspects of the cellular pharmacology of *N-l*-leucyldoxorubicin in human tumor cell lines

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Abstract—We have compared the cytotoxicity, incorporation and metabolism of doxorubicin (dox) and *N-l*-leucyldoxorubicin (leu-dox) in two human tumor cell lines in culture, the MCF-7 breast cancer line and the K562 leukemia line, and their dox-resistant counterparts. Dox was 3–4-fold more cytotoxic than leu-dox in the MCF-7 lines, and 7–10-fold in the K562 lines. This could be explained by differences in cell incorporation of the drugs, which differs by the same proportion as the cytotoxicities in the various cell lines, rather than by differences in biotransformation of leu-dox into dox, which is similar in all of the cell lines.

N-l-Leucyldoxorubicin (leu-dox*) is a new anthracycline which is currently being developed as a prodrug of doxorubicin (dox) for the purpose of improving its therapeutic index by reduction of its cardiac toxicity at equimyelotoxic doses [1]. In a recently published phase I trial, this equivalence of myelotoxicity was found at a dose ratio (leu-dox/dox) of 3 [2]. The question was raised as to whether the biotransformation of leu-dox to dox was strictly required for the activity of this analog or not. Several arguments for such a requirement were deduced from the phase I study already mentioned, especially the fact that at the maximum tolerated dose of leu-dox, the circulating levels of dox were similar to those obtained at the maximum tolerated dose of dox [2]. It was not clear, however, if the reduced toxicity of leu-dox was due to a low cellular uptake or to a limited biotransformation of the prodrug to the active species doxorubicin. We have therefore conducted a comparative *in vitro* study of leu-dox and dox in two human tumor cell lines, the MCF-7 breast cancer line and the K562 erythroleukemia line, which have been used for a long time as models for cytotoxicity evaluations. We have tried to correlate cytotoxicity to drug incorporation and biotransformation in these two cell lines and their doxorubicin-resistant variants, and to identify the reason for the lower cytotoxicity of leu-dox as compared to dox.

Materials and Methods

Chemicals. Leu-dox and its dihydroderivative *N-l*-leucyldoxorubicinol were kindly provided by the Medgenix group (Fleurus, Belgium). Dox and daunorubicin were generous gifts of Rhône-Poulenc-Santé (Vitry, France). All other reagents were of analytical grade.

Cell culture. The cell lines used in this study were MCF-7 human breast cancer cells [3] and their dox-resistant variant currently growing with 10 μM dox [4]; and K562 human erythroleukemia cells [5] and their dox-resistant variant currently growing with 0.3 μM doxorubicin [6]. The MCF-7 cells were grown as monolayers whereas K562 cells grew in suspension, both with RPMI 1640 medium supplemented with 10% fetal calf serum obtained from Seromed (Berlin, Germany), and maintained in humidified atmosphere containing 5% CO_2 at 37°. The cells were regularly replicated and growth curves frequently repeated to ensure the permanence of the growth characteristics of the culture.

Chemosensitivity evaluation. Depending upon growth characteristics, 1000–3000 cells were seeded in each well of 96-well plates. Two or three days later, incubations with dox or leu-dox were performed for 2 hr at appropriate concentrations ranging between 1.72 nM and 17.2 μM for both wild strains, between 17.2 and 1720 μM for the MCF-7 resistant strain, and between 0.0172 and 172 μM for K562 resistant strain. At the end of incubations, the medium was removed (after centrifugation in the case of K562 cells), the cells were washed with buffered saline and allowed to grow for 2 or 3 more days, depending upon the growth pattern of the lines. At this time, the surviving cells were estimated by MTT assay [7] and the IC_{50} values were calculated (drug concentrations responsible for 50% growth inhibition as compared to cells incubated without drug).

Drug incorporation and efflux. Drug incorporation experiments were designed to reproduce the conditions of exposure used for the determination of cytotoxicity. Cells were seeded in 10-cm^2 Petri dishes so as to obtain 2.5×10^6 cells/dish 3 days later. Drug was then added (1 or 2 μg/mL for sensitive cells and 10 or 32 μg/mL for resistant cells) and incubations were performed for various times (15 min–2 hr) to study drug uptake. At the end of the 2-hr incubations, the medium was removed, the cell monolayers washed and the cells reincubated further for various times (5 min–24 hr) to study drug efflux. After all incubations, with or without reincubation in drug-free medium, the cells were harvested in buffered saline by gentle stirring and collected in polypropylene tubes. After centrifugation, the cell pellet was immediately extracted with chloroform/methanol 4/1 after addition of daunorubicin to be used as an internal standard [8]. The organic phase was evaporated to dryness and reconstituted with 0.2 mL of the mobile phase of chromatography.

HPLC was performed on a Microbondapak-phenyl column purchased from Waters (30 × 0.39 cm) with a mobile phase constituted of acetonitrile (32 vol.) and a pH 4.0 0.1% ammonium formate buffer (68 vol.), at a flow rate of 3 mL/min, as described by Israel *et al.* [9]. Detection was achieved with a Hitachi F-1050 spectrofluorometer with excitation and emission wavelengths set at 436 and 592 mm, respectively. Under these conditions, the retention times of dox, leu-dox and daunorubicin were 2.5, 3.3 and 4 min, respectively.

From the results obtained, intracellular exposures were calculated as areas under the time–concentration curve

* Abbreviations: dox, doxorubicin; leu-dox, *N-l*-leucyldoxorubicin; AUC, area under the time–concentration curve.

Table 1. Comparative cytotoxicity and incorporation of dox and leu-dox in sensitive and resistant MCF-7 and K562 cells

	Dox		Leu-dox		Dox proportion after leu-dox exposure	
	IC_{50} (μM)	Incorporation (AUC 0–2 hr) (pmol/10^6 cells × hr)	IC_{50} (μM)	Incorporation (AUC 0–2 hr) (pmol/10^6 cells × hr)	AUC (%) 0–2 hr during drug exposure	AUC (%) 0–24 hr after drug removal
MCF-7	0.10 ± 0.02	739 ± 14	0.32 ± 0.13	458 ± 17	9.4	50.2
MCF-7R	117 ± 10	436 ± 55	417 ± 42	172 ± 65	10.0	30.3
K562	0.21 ± 0.04	408 ± 62	2.30 ± 1.42	71 ± 14	9.7	43.0
K562 R	3.44 ± 1.72	1013 ± 143	25.9 ± 10.1	141 ± 6	7.9	27.8

Results are means ± SD of three independent experiments performed in triplicate.

IC_{50} values were estimated after 2-hr exposures to various concentrations of the drug and regrowth during 2.5 cell cycles.

Drug concentrations were estimated in the cells after exposures lasting 15, 30, 60 and 120 min and expressed as AUCs by multiplying time by concentration. External drug concentration was either 1 (sensitive cells) or 10 (resistant cells) μg/mL.

The proportion of doxorubicin was calculated from AUCs either at the end of the 2-hr incubations, or at the end of the 24-hr post incubations.

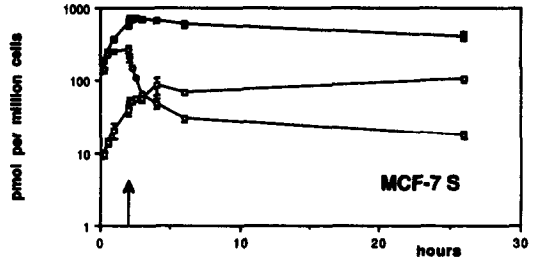

Fig. 1. Incorporation of dox (■) and leu-dox (○), and transformation of leu-dox into dox (□), in sensitive MCF-7 cells during and after a 2-hr exposure to the drugs. Results are means of three independent experiments performed in triplicate. Drugs were extracted from the cells either after 15-min–2-hr incubations at drug concentrations of 1 μg/mL; or after reincubation without drug for 5 min–24 hr following the 2-hr exposure indicated by an arrow. Extracts were analysed by HPLC as described in Materials and Methods.

(AUCs) by the trapezoidal rule. In cells treated with dox, this compound was the only one to be recovered, whereas in cells treated with leu-dox, both the original compound and dox were identified in the cell extracts.

Results and Discussion

Table 1 presents the IC_{50} values of dox and leu-dox in both human cell lines. The two lines differ greatly in their degrees of resistance to doxorubicin, the MCF-7 line being >1000-fold resistant, whereas the K562 line was only 15-fold resistant. The degree of resistance to leu-dox in both lines was quite similar to the degree of resistance to dox. When comparing the IC_{50} values of dox and leu-dox, it appears that they differ by a factor of around 3–4 in MCF-7 cells (sensitive and resistant) and 7–10 in both K562 lines, indicating that leu-dox was less active against the K562 lines than against the MCF-7 lines.

Figure 1 presents an example of the incorporation of dox and leu-dox during the 2-hr incubation, and of the subsequent efflux during the 24-hr post incubation. For such incubations, we have chosen different drug extracellular concentrations for sensitive and resistant cells because of the usual decrease in anthracycline incorporation in multidrug-resistant cells expressing P-glycoprotein [10]. Indeed, it was necessary to incubate resistant cells with 10-fold higher doses than sensitive cells to obtain intracellular concentrations of the same order of magnitude in sensitive and resistant cells. Cumulative incorporations are presented in Table 1 as AUCs (0–2 hr) and can be compared to the cytotoxicity data.

Concerning dox, there is a clear relationship between intracellular drug exposure and cytotoxicity in the sensitive lines: the IC_{50} is 2-fold lower in the MCF-7 line than in the K562 line and the incorporation 2-fold higher. This relationship is lost in resistant cells: the incorporation of dox is only 2.3-fold higher in K562 cells than in MCF-7 cells for an IC_{50} ratio of 34 between the two cell lines. This clearly indicates that intracellular drug concentration is not the only determinant for drug resistance in one (or both) cell lines, and that mechanisms other than enhanced drug efflux mediated by P-glycoprotein are likely to exist in the resistant cell lines. It is indeed well known that dox-resistant MCF-7 cells present additional mechanisms to the classical P-glycoprotein-mediated mechanism of resistance [4] and it is now generally admitted that resistance to doxorubicin is frequently multifactorial [11, 12].

Concerning leu-dox, it also appears that the amounts incorporated by the sensitive cells are in relation to cytotoxicity: there is a 6.5-fold higher intracellular exposure for the MCF-7 cells as compared to the K562 cells, and the IC_{50} is 7-fold lower for MCF-7 cells. As with dox, there is clearly no such relationship for resistant cells: total drug incorporations were similar in the two cell lines, whereas cytotoxicity was 16-fold lower in K562 cells than in MCF-7 cells.

When comparing leu-dox to dox incorporations in the K562 and MCF-7 cells, it appears that the ratio of intracellular exposures dox/leu-dox is about 2–3 in the MCF-7 lines and 6–7 in the K562 lines; these ratios are of the same order of magnitude as the IC_{50} ratios observed for the two drugs, suggesting that drug incorporation is at least one of the main determinants of leu-dox action.

Table 1 also presents the biotransformation of leu-dox to dox in the cell lines as AUC proportions of metabolite

either during drug exposure or during reincubation without drug. The formation of dox from leu-dox appears to be very minor during the 2-hr incubation, since dox represents 7–10% of the leu-dox incorporated in all cell lines. However, during the post incubations, there is a continuous transformation of leu-dox to dox such that the proportions of the two compounds are relatively similar. In the MCF-7 and K562 lines, the amount of dox formed from leu-dox was similar; this shows that the origin of the difference in cytotoxicity of leu-dox between MCF-7 and K562 cells is not the intracellular transformation of leu-dox into dox, but the uptake of leu-dox, which was lower in K562 cells than in MCF-7 cells. In all cases, dox appears as the likely cytotoxic species after leu-dox administration.

In conclusion, we have shown in this paper that leu-dox is incorporated to a lesser degree than dox in both human cell lines *in vitro*, and that this difference is responsible for a reduced efficacy of leu-dox as compared to dox, in both dox-sensitive and dox-resistant cells. Intracellular transformation of leu-dox to dox does not appear as a limiting step of leu-dox activity in these two human cell lines or their multidrug-resistant variants.

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