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SHORT COMMUNICATION

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Comparison of idarubicin and daunorubicin and their main metabolites regarding intracellular uptake and effect on sensitive and multidrug-resistant HL60 cells

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Abstract To study the effect of the main metabolites on the cytotoxic effect of daunorubicin and idarubicin in human HL-60 cells, drug-sensitive and multidrug-resistant HL60 cells were incubated with idarubicin and daunorubicin and their metabolites idarubicinol and daunorubicinol over a wide range of concentrations. The intracellular uptake of the drugs was determined by photofluorometry, and the cytotoxic effect in vitro was determined by a bioluminescence assay of intracellular adenosine triphosphate (ATP) after 4 days of culture in liquid medium. The effect of intracellular drugs was calculated from the incubation-concentration versus intracellular-uptake and cytotoxic-effect curves. The intracellular uptake of idarubicin was 6 times that of daunorubicin in drug-sensitive cells and 25 times higher in resistant cells. For idarubicinol as compared with daunorubicinol the corresponding factors were 25 and 7, respectively. As compared with the parent substances, the uptake of idarubicinol and daunorubicinol was 16% and 4%, respectively, in sensitive cells and 40% and >100%, respectively, in resistant cells. An intracellular concentration of 0.5 nmol/mg protein of both parent substances caused a 50% growth inhibition in drug-sensitive cells as compared with 10 nmol/mg protein for drug-resistant cells. For the metabolites an intracellular concentration of 0.4 nmol/mg protein of idarubicinol and 2.0 nmol/mg protein of daunorubicinol was required to inhibit cells' growth by 50% in drug-sensitive HL60 cells. In the resistant HL60 cells the corresponding values were 30 nmol/mg protein for idarubicinol and 40 nmol/mg protein for daunorubicinol. These results confirm that idarubicinol may significantly contribute to the clinical effect of idarubicin. However, in combination with previous results that have shown low intracellular concentrations of the metabolites in vivo, it appears that the pharmacokinetic properties of the mother substances provide the major explanation for the clinical effect of idarubicin.

Key words Idarubicin · Idarubicinol · Daunorubicinol · Intracellular uptake · In vitro effect

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Introduction

Anthracycline antibiotics are widely used as anticancer agents. Of several derivatives, idarubicin (4-demethoxydaunorubicin) has been most recently introduced for widespread clinical use. The equitoxic dose of idarubicin is one-fourth to one-fifth that of daunorubicin [1]. Preclinical studies indicate an incomplete cross-resistance to daunorubicin [4, 9]. Like daunorubicin, idarubicin has demonstrated clinical activity mainly in hematological malignancies, and several controlled clinical trials have shown that its antileukemic effect is equal to or better than that of daunorubicin [6, 8, 14, 25, 26]. Data also indicate a reduced chronic cardiotoxicity of idarubicin [9].

The plasma pharmacokinetic parameters of idarubicin are similar to those of daunorubicin except that the main metabolite, idarubicinol, appears more rapidly and reaches higher concentrations as compared with daunorubicinol [18, 20–22]. Within a few minutes the concentration of idarubicinol is higher than that of the parent substance, and whereas idarubicin is eliminated with a terminal elimination half-life ($t_{1/2}$) of 15–22 h, idarubicinol is retained much longer in plasma ($t_{1/2}$ 40–80 h).

The prolonged and high concentrations of idarubicinol are believed to contribute to the difference in clinical activity as compared with daunorubicin. However, the main target for anthracyclines is situated within the nucleus of leukemic cells. We have previously shown that there is no simple correlation between the plasma pharmacokinetics and the intracellular drug concentrations [16, 17, 23]. In a recent study we made a direct comparison of the intracellular pharmacokinetics in vivo of daunorubicin and idarubicin given simultaneously to the same patients. We showed that the intracellular uptake of idarubicin was considerably higher than that of daunorubicin, whereas the uptake of the metabolites was low and probably not decisive for the clinical effect [24].

In the present study we wanted to elucidate further the role of idarubicinol in the antileukemic effect of idarubicin. We studied the intracellular uptake and effect in vitro of idarubicin and daunorubicin and their main metabolites in drug-sensitive and multidrug-resistant HL60 cells. The results support the idea that the pharmacokinetic properties of the mother substance provide the primary explanation for the clinical activity.

Materials and methods

HL60 cells

Two sublines of HL60 cells were used, a drug-sensitive wild type, HL60S, and a multidrug-resistant subline, HL60R. The latter was established by continuous exposure to increasing concentrations of doxorubicin and has been extensively characterized [10]. The 50% growth-inhibitary concentration (IC_{50}) is 1300 times higher for daunorubicin and 280 times higher for idarubicin in HL60R as compared with HL60S. HL60R cells express increased levels of type 1 multidrug resistance (*mdr1*) mRNA and are positive for glycoprotein P170. The cross-resistance pattern is that of classic multidrug resistance, and the cells show reduced intracellular uptake and increased, verapamil-reversible, efflux of doxorubicin. Both cell lines were kept in a medium consisting of RPMI 1640 supplemented with 10% fetal calf serum and 1% L-glutamine. The medium was changed and the cells were resuspended at a concentration of 2×10^5 cells/ml every 5th to 7th day. Cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Incubations

Idarubicin, idarubicinol (both from Farm-Italia Carlo Erba, Milan, Italy), daunorubicin, and daunorubicinol (both from Rhone-Poulenc Rhorer) were diluted in phosphate-buffered saline (PBS) to 10 times the final incubation concentrations. Cell suspension (1.8 ml) was incubated for 1 h at 37°C with 0.2 ml of anthracyclines in a gently shaking bath. All incubations were performed in duplicate. Final concentrations used for HL60S incubations were as follows: idarubicin 0.002–2.0 μM, idarubicinol 0.005–20 μM, daunorubicin 0.01–5.0 μM, and daunorubicinol 0.05–20 μM. For HL60R the final concentrations were as follows: idarubicin 0.05–50 μM, idarubicinol 0.2–50 μM, daunorubicin 2.0–1000 μM, and daunorubicinol 50–1000 μM. All experiments were repeated at least once.

Intracellular uptake

For in vitro uptake studies in leukemic and normal bone marrow cells, we used a cell concentration of 1.0×10^6 cells/ml. The incubations were terminated by the addition of 5 ml of ice-cold PBS and the cells were thereafter kept on ice. Cells were washed twice in PBS at 4°C and frozen at −20°C until analyzed. After thawing, the cells were sonicated for 20 s at 50 W with a Branson B-12 sonicator (Branson Sonic Power Company, Danbury, Const.) and the drugs were extracted with trichloroacetic acid (TCA, 27%). The drugs were assayed by photofluorometry as previously described using a Shimadzu model RF-510 spectrofluorometer (excitation and emission wavelengths 485 and 560 nm, respectively) [2]. Anthracycline concentrations in each sample were determined by comparison with identically treated standard solutions and related to the amount of cell protein determined according to Lowry et al. [13].

In vitro effect

The cytotoxic effect was determined by a bioluminescence assay of cellular adenosine triphosphate (ATP) as previously described [19]. The ATP-monitoring reagent and the ATP standard used were supplied by Bio Orbita (Turku, Finland). After incubation of leukemic cells at a cell concentration of 1.0×10^5 cells/ml with anthracyclines as described above, the cell suspension was centrifuged (400 *g* for 10 min) and the medium was removed. Without a wash, the cells were resuspended in fresh medium [2 ml of RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine in each tube] and cultured for 4 days at 37°C in a humidified incubator containing 5% CO_2 (ASSAB, T-303). The bioluminescence assay was performed automatically in a Bio Orbit photometer (Turku, Finland). Results were given as nanomoles of ATP per sample, and the effect was calculated as the percentage of living cells in a drug-treated sample as compared with a drug-free control. The incubation concentration required to inhibit cell growth to 50% of the untreated control value (IC_{50}) was achieved from the incubation-concentration versus effect curve.

Effect of intracellular drugs

The intracellular drug concentration required to inhibit cell growth to 50% was calculated by transforming the IC_{50} value, calculated as described above, to the incubation-concentration versus intracellular-uptake curve.

Results

Intracellular uptake of parent substances and metabolites

The intracellular uptake of idarubicin was higher than that of daunorubicin over a wide range of incubation concentrations for both HL60S and HL60R cells as shown in Fig. 1. In HL60S, the difference was most pronounced in the interval ranging from 0.2 to 1.0 μM, where the uptake of idarubicin was 4–10 times that of daunorubicin. In HL60R the uptake of idarubicin was more than 20 times that of daunorubicin at all concentrations tested.

As for the parent substances, the intracellular uptake of idarubicinol was higher than that of daunorubicinol

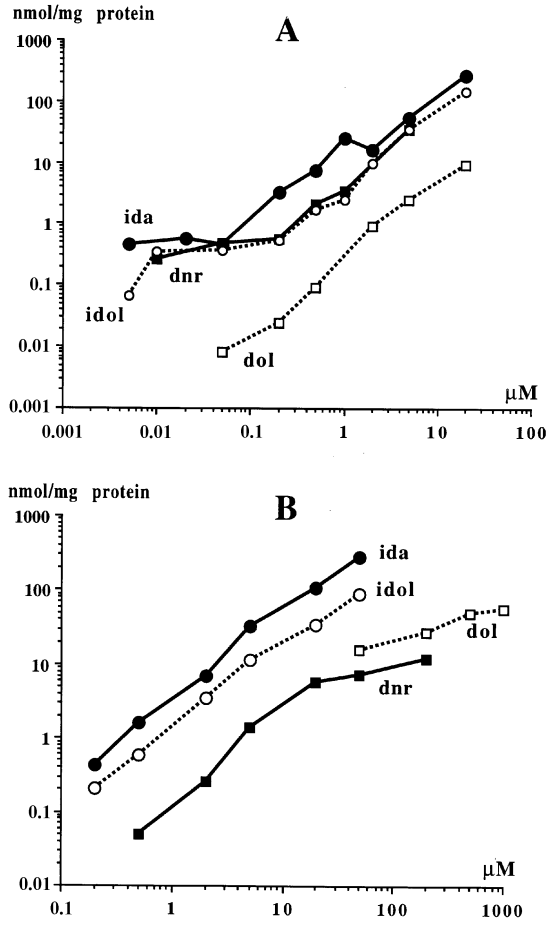

Fig. 1 A, B Intracellular uptake of idarubicin (ida, *black circles*), idarubicinol (idol *white circles*), daunorubicin (*dnr*, *black squares*), and daunorubicinol (*dol*, *white squares*) in **A** drug-sensitive and **B** multidrug-resistant HL60 cells after incubation for 1 h at different concentrations

over a wide range of concentrations (Fig. 1). For HL60S this difference exceeded a factor of 10 at all concentrations tested. Due to the low cytotoxic effect on HL60R cells, the incubation concentrations were found to overlap only at a concentration of 50 μ*M*, where the uptake of idarubicinol was 5 times that of daunorubicinol.

The intracellular uptake of the metabolites was consistently lower than that of the parent substances. At an incubation concentration of 0.2 μ*M* the uptake in HL60S cells was 3.2 nmol/mg protein for idarubicin, 0.53 nmol/mg protein for idarubicinol, 0.56 nmol/mg protein for daunorubicin, and 0.025 nmol/mg protein for daunorubicinol. Thus, the uptake of the metabolites was less than 20% of that of the parent substances. In HL60R cells this difference was reduced. The uptake of daunorubicinol was similar to that of daunorubicin, whereas the uptake of idarubicinol was 50% of that of idarubicin.

Table 1 Intracellular concentrations (nmol/mg protein) of daunorubicin (*dnr*), idarubicin (*ida*), daunorubicinol (*dol*), and idarubicinol (*idol*) that caused a 50% inhibition of the growth of HL 60S and HL60 R cells

	dnr	ida	dol	idol
HL 60S	0.5	0.5	2.0	0.4
HL 60R	10	10	40	30

In vitro effect of parent substances and metabolites

Idarubicin was consistently more toxic to both HL60S and HL60R cells. The IC_{50} value in HL60S cells was 0.01 μ*M* for idarubicin as compared with 0.17 μ*M* for daunorubicin. In comparison, the IC_{50} value in HL60R cells was 200 times higher for idarubicin (2.1 μ*M*) and 1000 times higher for daunorubicin (165 μ*M*).

Also, idarubicinol was consistently more toxic to both HL60S and HL60R cells than was daunorubicinol. The IC_{50} value in HL60S cells was 0.36 μ*M* for idarubicinol as compared with 3.9 μ*M* for daunorubicinol. In comparison, the IC_{50} value in HL60R cells was 40 times higher for idarubicinol (15 μ*M*) and 90 times higher for daunorubicinol (350 μ*M*). Thus, the equitoxic incubation concentration of the metabolites was 20–30 times that of the parent substances in HL60S cells but only 2–7 times that of the parent drugs in HL60R cells.

Effect of intracellular parent substances and metabolites

At the incubation concentration that resulted in 50% growth inhibition (IC_{50}) the intracellular concentration of both daunorubicin and idarubicin was 0.5 nmol/mg protein in HL60S cells and 10 nmol/mg protein in HL60R cells (Table 1). The intracellular uptake at the IC_{50} of idarubicinol and daunorubicinol in HL60S cells was 0.4 and 2.0 nmol/mg protein, respectively (Table 1). For HL60R cells the corresponding values were 30 nmol/mg protein for idarubicinol and 40 nmol/mg protein for daunorubicinol.

Discussion

All commonly used anthracyclines are mainly metabolized by a cytoplasmic reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aldo-keto reductase to a glycoside metabolite (i.e., doxorubicinol, epirubicinol, daunorubicinol) that has antitumor activity [3]. The plasma concentrations of e.g., daunorubicinol exceed those of the parent substance within a few minutes, and the metabolite is retained in plasma much longer [18, 20–22]. Due to

their reduced lipid solubility, the cellular uptake of these metabolites is low [17] and they are generally not considered to be of importance for the clinical effect of the drugs. However, as compared with the other anthracyclines in clinical use, the plasma concentrations of idarubicinol are higher and the retention is longer. Detectable concentrations of idarubicinol can be seen for up to 3 days after administration [22]. Studies in cell lines have shown that the cytotoxic effect of idarubicinol is stronger than that of the other anthracycline metabolites [11], and in one study on OCI AML-3 cells the toxicity of idarubicinol was similar to that of idarubicin [12]. On the basis of such data it has been postulated that idarubicinol may significantly contribute to the clinical effect of idarubicin.

In the present study we showed that the intracellular uptake of idarubicin was considerably higher than that of daunorubicin, which is in accordance with the results of other studies [5, 7]. The uptake of both metabolites was low. The IC_{50} value determined for daunorubicin and daunorubicinol was more than 10 times that recorded for idarubicin and idarubicinol, respectively. This is also in accordance with the results of other studies, whereby the difference in effect has varied, depending on the cell lines that are used [11, 12]. What we have done is to correlate the cytotoxicity of parent substances and metabolites to the intracellular uptake. By this type of analysis we demonstrated that in drug-sensitive and multidrug-resistant HL60 cells, both idarubicin and daunorubicin were equitoxic at the same intracellular concentrations. As for the metabolites, intracellular concentrations in the same range as those of the parent substances were required to achieve the same cytotoxic effect, although idarubicinol was more toxic.

Clinically, the equitoxic dose of idarubicin is 20–25% of that of daunorubicin. In a previous study we treated leukemic patients with a mixture of idarubicin and daunorubicin [24]. Although idarubicin was given as one-fifth of the mixed dose, the intracellular peak concentration of this drug was 70% of that of daunorubicin. The intracellular concentration of the metabolites was approximately 1/15th of the peak concentration of the parent substances, but it remained constant during the 24-h study period. When these in vivo findings are compared with the present results regarding the toxicity of intracellular metabolites, it appears as if the contribution of the metabolites to the clinical effect of idarubicin is small. However, it cannot be ruled out that the persistence of intracellular metabolites at low concentrations contributes to the clinical effect.

It can be argued that a 1-h period of incubation is too short to yield the full effect of idarubicinol since the in vivo retention of this drug in plasma is prolonged. If so, this should to a great extent also apply to daunorubicinol. Besides, the analysis of the effect of intracellular metabolites is not affected by incubation conditions.

Other studies have shown that idarubicin exhibits reduced cross-resistance of various degrees to daunorubicin [4, 9]. Doxorubicin-selected resistant HL60 cells that do not express P170 [15] and HL60 cells that are equally sensitive to idarubicin and idarubicinol have been described [12]. Our HL60R cells are highly resistant to all anthracyclines but the cross-resistance to idarubicin is not complete. Such differences in results concerning the degree of cross-resistance and the relative toxicity of the metabolites could be due to the cell line studied and to how the cells were selected for resistance. In our HL60 cells the intracellular concentrations that induced a 50% growth reduction were exactly the same for idarubicin and daunorubicin in both sublines. Interestingly, this concentration was 20 times higher in the resistant cells. A similar reduced effect of the intracellular metabolites was seen. These findings indicate that the resistance is not due to drug-transport mechanisms alone.

In conclusion, this study shows that the effect of the previously described low concentrations of intracellular idarubicinol is weak but possibly contributory to the clinical effect of idarubicin. The results support the idea that the pharmacokinetic properties of unmetabolized idarubicin provide the principal explanation for the observed difference in clinical effect as compared with daunorubicin.

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