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Uptake and intracellular distribution of idarubicin in secondary cultures of normal and neoplastic urothelium

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Abstract This study analyzes the uptake and endocellular distribution of idarubicin (IDA) in normal and neoplastic urothelial secondary cultures in relation to the changes in concentration and time of exposure. The urothelial lines were isolated by Freshney's method from biopsy fragments taken from five patients with superficial bladder cancer. Pharmacological experiments were carried out on subcultures previously immunophenotypically characterized and did not exceed ten passages. The uptake and endocellular distribution of IDA was analyzed by densitometric image analysis on cells treated for 10, 20, 30 and 60 min and 2 h with scalar dosages from 10 ng/ml to 2430 ng/ml. Microscopic observations and densitometric analyzes revealed that in the cells treated with IDA, fluorescence was higher in the cytoplasm compared to the nucleus and increased with the change in dosage. Moreover, densitometric data showed that IDA uptake in the first 20 min was higher in the neoplastic cells, but after that period its behavior became heterogeneous at 30 and 60 min, while at 2 h there was an inversion of the trend. These results suggest that the in vitro cytotoxicity should be evaluated in order to verify whether the elevated uptake of IDA in the first 20 min of treatment is really correlated to a more elevated toxicity in the neoplastic cells with respect to the normal cells. This is presently under investigation.

Key words Idarubicin · Urothelial secondary cultures · Intracellular distribution · Densitometric image analysis

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Idarubicin (4-demethoxydaunorubicin, IDA) is an analogue of daunorubicin. The only difference between the two is the substitution of the methoxyl group with a hydrogen atom in the C-4 position of the D ring of the aglycon group. This structural modification supplies to IDA an elevated lipophilia which increases its capacity of penetration in cells, widens its range of action and increases its therapeutic index with respect to other anthracyclines [1, 7, 9]. Recently, Mross et al. [11] suggested using this drug even in topical treatment of the superficial forms of bladder cancer, where endovesical chemotherapy has been widely used as prophylactic treatment in order to reduce the recurrence rate after transurethral resection (TUR) [8]. These authors, after having intravesically administered IDA in 33 patients affected by bladder tumors, noticed higher drug levels in the tumor tissue than in the normal mucosa. These results are of great clinical interest even if the evaluation of the IDA uptake was carried out on the whole fragment of mucosa and little attention was paid to the levels reached by this drug in only the urothelium. On the other hand, unlike what has been reported in the literature [1–3, 7, 9] about other types of cells, no further study has been carried out up to now to determine the absorption and the endocellular distribution of IDA in the urothelial cells. With this work we intended to take into consideration this aspect by studying the uptake and endocellular distribution of IDA by means of densitometric image analysis in neoplastic and normal urothelial secondary cultures with varying drug concentrations and exposure times.

Materials and methods

Chemical compounds

Idarubicin (Zavedos) was kindly supplied by Farmitalia (Italy). Stock solutions were prepared in sterile physiological solution for both drugs and kept in the dark at −20°C. These were diluted with the culture medium to the final concentrations, immediately prior to use.

Patients

Neoplastic and normal biopsy samples were taken from five patients with superficial bladder cancer. These were selected regardless of the type of lesion (primary or recurrent, mono- or multifocal, grading) and who had not previously undergone any chemotherapy or endovesical bacillus Calmette Guérin (BCG) therapy. These patients, three men and two women, median age 72 years (range 68–80 years), presented multiple papillary neoformations at diagnosis. Urinary cytology was positive in three patients and negative in two. They all underwent endoscopic resection and the results of the histologic examination were TaG1 in three and TaG1-G2 and T1aG2 in the remaining two [16]. Informed consent was obtained from all patients before collecting the biopsy samples.

Cell cultures

Biopsies were cultured using a modified Freshney's method [4]. The tissue samples were mechanically divided into fragments of 1 mm^3 and placed on a feeder layer consisting of fibroblastic mouse cells 3T3-J2 pretreated for 2 h with mitomycin C at a final concentration of 10 μg/ml. The secondary cultures were prepared when the colonies reached maximum growth, between the 7th and the 15th day of incubation, by treating them with a 0.05% trypsin and 0.02% ethylenediaminetetraacetate (EDTA) solution. Ham's F-12/DMEM high-glucose (1:1 mixture, 3151 mg/l glucose, Irvine Scientific), buffered with Hepes and supplemented with 10% fetal calf serum (Seromed), linoleic acid-albumin supplement 500 mg/l (Sigma), glutamine 2 mM, pyruvic acid 1 mM and antibiotics, was used as culture medium for both the primary and the secondary cultures. After suitable expansion, at the third or fourth passage, the cell lines obtained were frozen in complete F-12/DMEM containing 10% dimethylsulfoxide (DMSO). The pharmacological experiment on the subcultures between the fifth and tenth passages was preceded by phenotypic characterization of the lines. This showed that all lines, regardless of their normal or neoplastic origin, were cytokeratin positive and therefore epithelial (unpublished data).

Assessment of uptake and intracellular distribution

Cells, resuspended in complete culture medium at a concentration of 1×10^5 cell/ml, were seeded on 24-well plates and incubated at 37°C. After 22 h they were treated with drug for 10, 20, 30 and 60 min and 2 h with the following dosages: 10, 30, 90, 280, 810 and 2430 ng/ml. At the end of each incubation time, the treated cultures were washed with cold (+4°C) phosphate-buffered saline (PBS) to eliminate any excess drug, and were subjected to densitometric analysis under the optical microscope with ultraviolet light. Duplicates of each dosage were prepared for testing. Two wells of untreated cells were included in each treatment time and used as control to exclude any possibility of cellular self-fluorescence and another two with just culture medium were used as blanks to remove the background fluorescence. A Zeiss Axiovert 100 inverted microscope was used fitted with epi-fluorescence and a 450/490-nm exciter filter, FT510 chromatic beam splitters and an LP520 barrier filter, connected by a videocamera to a computerized image analysis system (Vidas21, Zeiss). This device gave readings of the following parameters: (a) mean cellular emission (CELL.E), (b) mean cytoplasmic emission (CYTO.E) and (c) mean nuclear emission (NUCL.E). The quantity of these emissions was evaluated as mean transmittance per square micrometer. At least 50 cells/treatment, for each cell line, were analyzed.

Statistical analysis

The image analysis data were evaluated by the Kruskal-Wallis and Mann-Whitney tests, taking $P < 0.05$ as the level of significance [6].

Results

Assessment of intracellular distribution and uptake

Microscopic observations revealed that the cells treated with IDA were fluorescent, whereas the control cells were negative. Fluorescence was higher in the cytoplasm than in the nucleus and increased with the change in dosage and exposure time. Its distribution also changed with the treatment time: at 10 and 20 min it was spread uniformly in the cytoplasm (Fig. 1), but after 30 min it was arranged in granules in the cytoplasm mostly around the nucleus, plus particularly visible spots along the cell edges and at the pseudopodia (Fig. 2). In treated

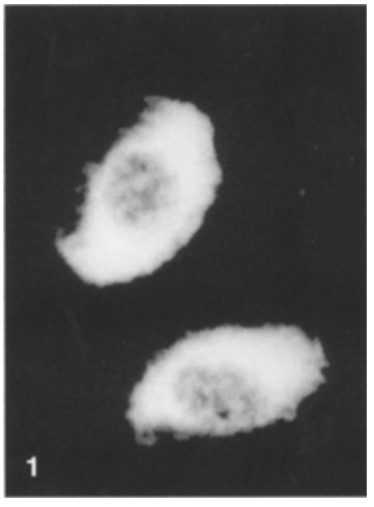

Fig. 1 Cells treated with 270 ng/ml idarubicin for 10 min. The fluorescence caused by IDA appears homogeneously distributed in the cytoplasm, × 400

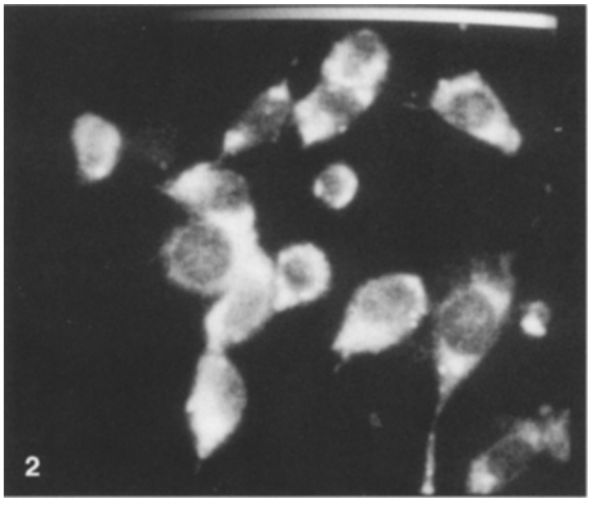

Fig. 2 Cells treated with 270 ng/ml idarubicin for 60 min. The fluorescence is arranged in granules distributed around the nucleus, along the cell edges and the pseupodia, × 200

cultures, the uptake was evaluated by densitometric analysis only for dosages exceeding 90 ng/ml, as the intensity of fluorescence was always lower than the sensitivity levels of our image analyzer for cultures exposed to concentrations of 10 ng/ml and 30 ng/ml and those treated with 90 ng/ml for only 10 min.

The results of the densitometric image analysis carried out on the normal and neoplastic lines underwent the following statistical comparisons:

1. Within each type of treatment between CELL.E, CYTO.E and NUCL.E (comparison A).
2. Within each time of treatment between the mean values at the different concentrations of IDA for each single parameter of emission (comparison B).
3. Within the single treatments (same dosages and times) between the neoplastic and the normal lines (comparison C).

Comparison A

Both in the neoplastic and the normal lines the comparison of the CELL.E, CYTO.E and NUCL.E mean values showed that the first two parameters were significantly higher ($P < 0.05$) than NUCL.E. The 20-min treatment with 90 ng/ml idarubicin was an exception in that only CELL.E was significantly higher ($P < 0.05$) in the neoplastic lines, while in the normal lines there was no statistically significant difference between the mean values of these three parameters.

Comparison B (see Figs. 3, 4)

These comparisons showed that, within each time of treatment, the increase in concentration of IDA induced a significant increase ($P < 0.05$) in the mean values of all three emissions, in both the neoplastic and the normal cells. However, the comparisons between the mean values of CELL.E, CYTO.E and NUCL.E at 30 min, obtained from cells treated with concentrations of 90 ng/ml and 270 ng/ml, were an exception in the normal cells: in fact they were not significant ($P > 0.05$).

Comparison C (see Table 1)

These comparisons show that the mean emission values of the neoplastic cells exposed to all dosages for 10 and 20 min were significantly higher ($P < 0.05$) than those of normal cells. At 30 min the comparisons revealed a reversal of tendency of the emission values. In fact, at a dosage of 90 ng/ml, neoplastic cell emissions were significantly lower ($P < 0.05$) than those of normal cells, while at dosages of 270, 810 and 2430 ng/ml, the densitometric parameters were not signficantly different between the two types of cells ($P > 0.05$). At 60 min none of the densitometric parameters of the neoplastic lines differed significantly from those of the normal lines at the dosage of 90 ng/ml ($P > 0.05$), but they were significantly higher ($P < 0.05$) at dosages of 270 and 810 ng/ml (except for NUCL.E at the dosage of 810 ng/ml), while at 2430 ng/ml they were lower

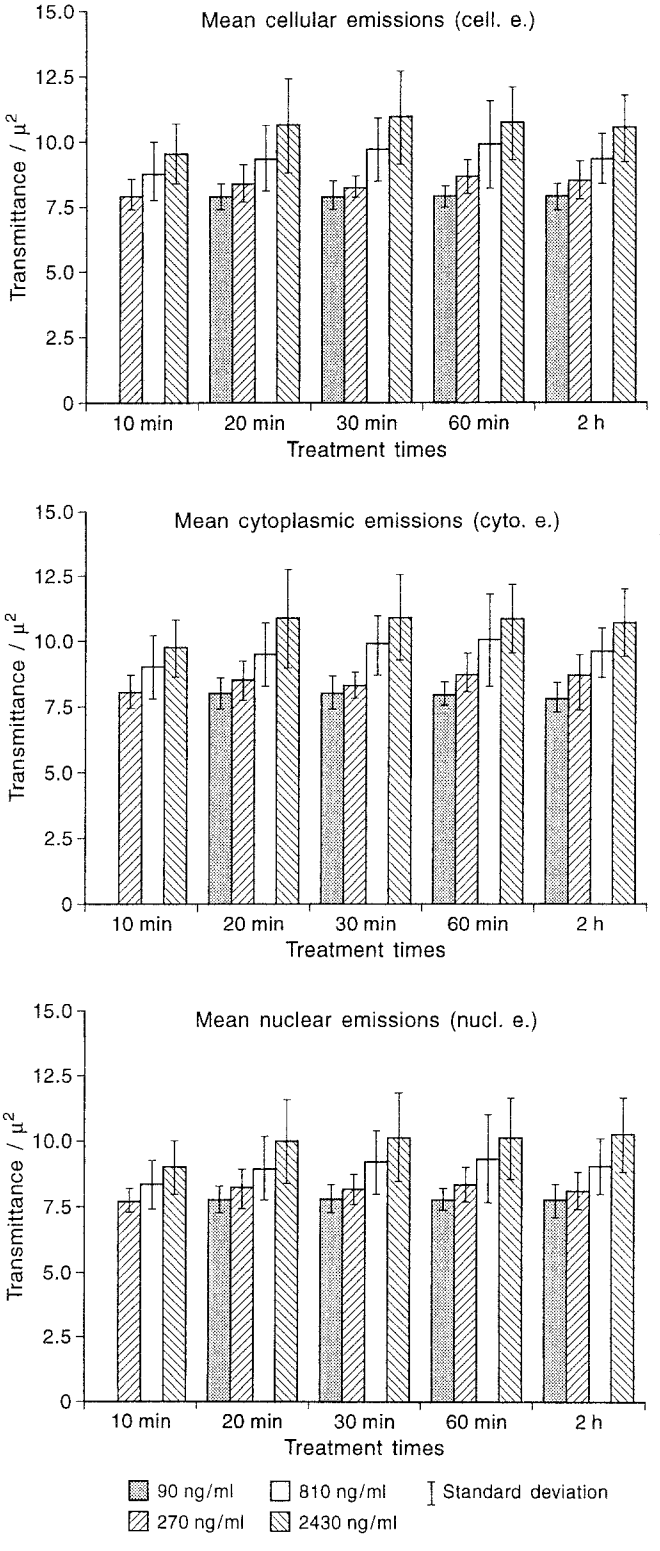

Fig. 3 Variations within each time of treatment of the mean cellular emissions (CELL.E), mean cytoplasmic emissions (CYTO.E) and mean nuclear emissions (NUCL.E) of neoplastic lines at the different concentrations of idarubicin

($P < 0.05$). The CELL.E, CYTO.E and NUCL.E of the neoplastic cells at 2 h were lower than those of the normal cells ($P < 0.05$), except at the dosage of 270 ng/ml.

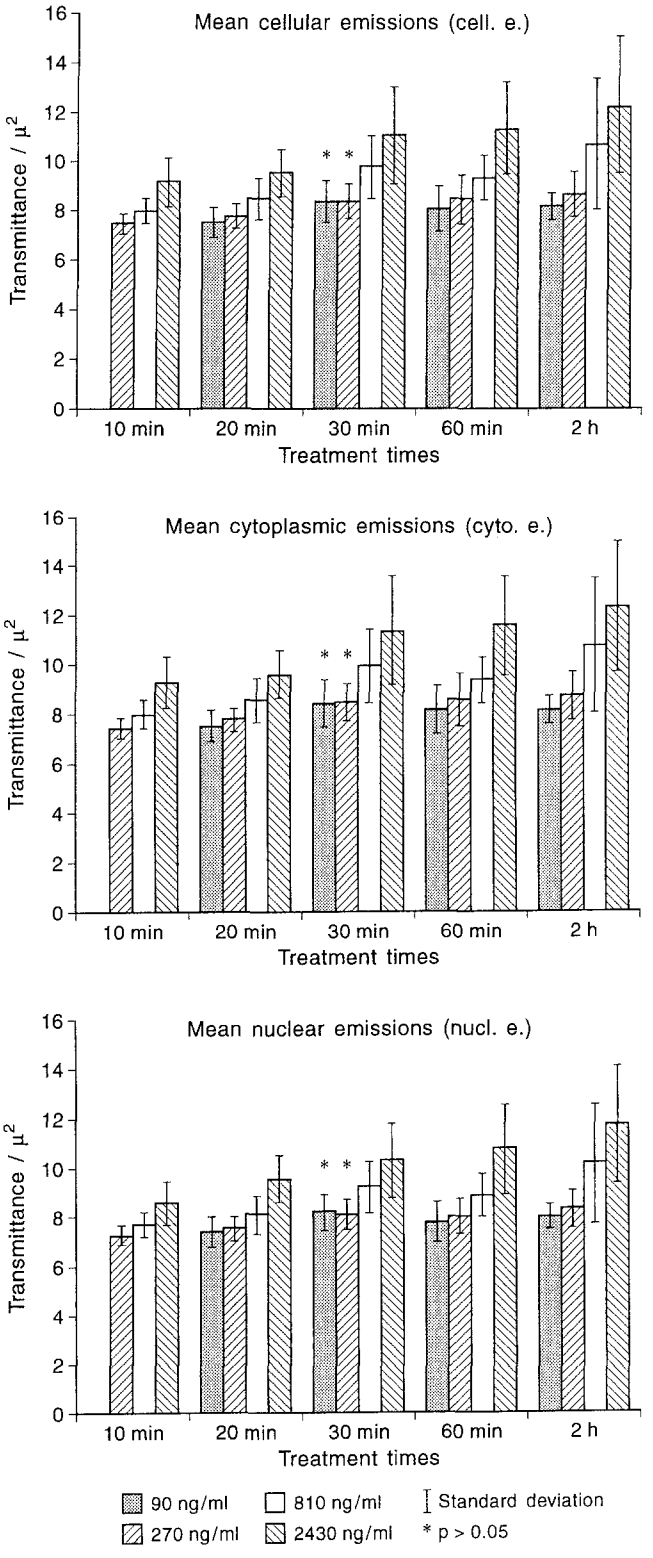

Fig. 4 Variations within each time of treatment of the mean cellular emissions (CELL.E), mean cytoplasmic emissions (CYTO.E) and mean nuclear emissions (NUCL.E) of normal lines at the different concentrations of idarubicin

Discussion

In this work the fluorescent properties, shown by all the anthracyclines when excited with ultraviolet light, has enabled the distribution of IDA inside the urothelial cells and their uptake to be analyzed. Indeed, treatment with IDA causes high fluorescence in the normal and neoplastic cells. In both types of cells its intensity is higher in the cytoplasm than in the nucleus and gradually grows with the increase in dosage. These microscopic observations and the relative densitometric data indicate that the absorption modality of IDA in the urothelial cells in secondary culture is analogous to those highlighted in the promyelocytic leukemia cell line HL 60 and in the human peripheral lymphocytes by Giesler et al. [5], in the B-cell lymphoma cell line DoHH2 by Smith et al. [14] and in the chronic myeloid leukemia cell line K-562 by Minderman et al. [10]. In particular, even in the urothelial cells, the passage of IDA through the different cellular compartments is influenced by the extracellular concentration of the drugs and this suggests that its uptake occurs for passive diffusion. This is in agreement with the correlation between lipophilia and intracellular accumulation of anthracyclin, which has already been reported by Hollingshead et al. [9], Goebel [7] and Facchetti et al. [3]. Moreover, the highest accumulation of IDA in the cytoplasm of our cells compared to the nucleous observed during all treatments, as well as its different fluorescent patterns in the cytoplasm when incubation exceeded 30 min, indicates that its uptake is also influenced by other absorption mechanisms. Actually, after prolonged exposure, the presence of luminous spots along the cell edges and in the pseudopodia as well as the grouping of fluorescent granules in the perinuclear zone, where the cytological stains (hematoxylin-eosin) have highlighted the ergastoplasm, lead to the presumption that IDA may accumulate in membranous type cytoplasmic structures such as the endoplasmic reticulum and the vacuoles. This hypothesis is in agreement with the observations of Sehested et al. [13]. Indeed, in the pleiotropic ascitic lines of Ehrlich and in the P388 line treated with alkaloids of the vinca group and with daunorubicin, they observed a significant increase in size of the endosome compartment connected with a noticeable increase in the endo-exocytotic traffic and with the drug-resistance expression. Also Toffoli et al. [15] showed the predominating cytoplasmic compartmentation of IDA in LoVo lines, whether IDA-resistant or not. On the contrary, these authors noted that daunorubicin (DAU) accumulated in the cytoplasm of the DAU-resistant but not in the DAU-sensitive LoVo lines. The results of the comparisons in the single treatments between the neoplastic and the normal lines are of great interest. IDA tends to accumulate more in the neoplastic cells in the first 20 min. After such a period its behavior becomes nonhomogeneous at 30 min and at 60 min, while after 2 h of incubation there is an inversion of the tendency. The highest uptake of IDA, which we noticed in the neoplastic ur-

Table 1 Emission parameter comparisons within the single treatments (same dosages and times) between the normal and neoplastic lines (means ± SD)

		90 ng/ml			270 ng/ml			810 ng/ml			2430 ng/ml		
		Normal	Neoplastic		Normal	Neoplastic		Normal	Neoplastic		Normal	Neoplastic	
10 min	CELL.E	–	–		7.4276 ± 0.4281	8.0101 ± 0.5930	*	7.9608 ± 0.5274	8.8876 ± 1.1660	*	9.1628 ± 1.0011	9.6047 ± 1.1332	*
	CYTO.E	–	–		7.4812 ± 0.4382	8.0307 ± 0.5937	*	8.0279 ± 0.5225	8.9822 ± 1.1980	*	9.2894 ± 1.0558	9.6338 ± 1.1043	*
	NUCL.E	–	–		7.2824 ± 0.4103	7.7317 ± 0.4607	*	7.6992 ± 0.4735	8.3208 ± 0.9042	*	8.5864 ± 0.8816	8.9918 ± 1.0508	*
20 min	CELL.E	7.5082 ± 0.6235	7.9329 ± 0.5687	*	7.7415 ± 0.4987	8.4323 ± 0.7637	*	8.4513 ± 0.8580	9.4419 ± 1.2254	*	9.5428 ± 0.9451	10.7128 ± 1.1853	*
	CYTO.E	7.5393 ± 0.6252	7.9513 ± 0.5795	*	7.7972 ± 0.4971	8.4763 ± 0.7671	*	8.5604 ± 0.8969	9.4725 ± 1.2154	*	9.6143 ± 0.9838	10.8362 ± 1.1905	*
	NUCL.E	7.4138 ± 0.6044	7.7838 ± 0.5102	*	7.5397 ± 0.4785	8.1996 ± 0.7576	*	8.0992 ± 0.8038	8.9639 ± 1.180	*	9.0765 ± 1.1800	9.9783 ± 1.5739	*
30 min	CELL.E	8.3619 ± 0.8613	7.9694 ± 0.5914	*	8.3773 ± 0.7032	8.3048 ± 0.4536	NS	9.7726 ± 1.3203	9.7676 ± 1.1771	NS	11.0826 ± 1.9937	10.9858 ± 1.8043	NS
	CYTO.E	8.4499 ± 0.9411	8.0094 ± 0.6108	*	8.4683 ± 0.7699	8.3117 ± 0.4670	NS	9.9684 ± 1.4795	9.8209 ± 1.1360	NS	11.3589 ± 2.2272	10.8893 ± 1.6784	NS
	NUCL.E	8.168 ± 0.7551	7.7973 ± 0.5287	*	8.0903 ± 0.5220	8.16 ± 0.5061	NS	9.1729 ± 1.0553	9.2105 ± 1.2016	NS	10.2505 ± 1.4942	10.1335 ± 1.6971	NS
60 min	CELL.E	8.0662 ± 0.9104	7.9631 ± 0.4232	NS	8.4474 ± 1.0144	8.7299 ± 0.6609	*	9.3191 ± 0.9086	9.9764 ± 1.7375	*	11.3603 ± 1.9164	10.7897 ± 1.4163	*
	CYTO.E	8.1792 ± 0.9767	8.011 ± 0.4321	NS	8.5746 ± 1.0619	8.7996 ± 0.6745	*	9.3894 ± 0.9300	10.0723 ± 1.7568	*	11.5718 ± 1.9978	10.8866 ± 1.3686	*
	NUCL.E	7.7875 ± 0.7816	7.7852 ± 0.4254	NS	7.9895 ± 0.7434	8.3523 ± 0.6476	*	8.8345 ± 0.8618	8.3459 ± 1.6930	NS	10.7086 ± 1.830	10.1151 ± 1.5467	*
2 h	CELL.E	8.147 ± 0.5659	7.9074 ± 0.5450	*	8.663 ± 0.9224	8.5657 ± 0.7848	NS	10.6914 ± 2.6597	9.4374 ± 0.9383	*	12.2628 ± 2.7722	10.6077 ± 1.2946	*
	CYTO.E	8.1835 ± 0.5799	7.9088 ± 0.5296	*	8.7358 ± 0.9679	8.6547 ± 0.8153	NS	10.7978 ± 2.703	9.5552 ± 0.9424	*	12.3501 ± 2.6659	10.6816 ± 1.3358	*
	NUCL.E	7.9666 ± 0.5304	7.7593 ± 0.6078	*	8.2783 ± 0.7706	8.1177 ± 0.6988	NS	10.1274 ± 2.3832	9.0526 ± 1.0677	*	11.6973 ± 2.3824	10.2511 ± 1.4078	*

* significant $P < 0.05$, *NS* not significant ($P > 0.05$)

othelial cells with respect to the normal cells at all dosages in the first 20 min, is in agreement with the results obtained in vivo by Mross et al. [11]. This confirms the validity of their study even if they evaluated IDA uptake in tissue fragments. These authors determined the total quantity of drug present in the tissues without discriminating between the accumulation of IDA in the urothelial component and its amount in the stromal ones. However, the study of phase I conducted by Schultze-Seemann et al. [12] showed that the single instillations of IDA for the duration of 1 h at the doses proposed by Mross et al. produce serious alterations in the normal urothelium accompanied by evident clinical symptoms of chemocystitis in over 50% of the patients. Our results in Table 1 suggest that the in vitro cytotoxicity should be evaluated in order to verify whether the elevated uptake of IDA in the first 20 min of treatment is really correlated with a more elevated toxicity in the neoplastic cells with respect to the normal ones. This is presently under investigation.

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