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Subcellular Distribution of Doxorubicin: Comparison of Fatty Acid-Modified and Unmodified Cells (42760)

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Abstract. We have examined the subcellular localization of doxorubicin and evaluated the effect of fatty acid modification on specific intracellular localization. L1210 leukemia cells enriched with docosahexaenoic acid (22:6) or oleic acid (18:1) were incubated with radiolabeled or unlabeled doxorubicin. After equilibration the cells were ruptured and the subcellular fractions were isolated by differential centrifugation and sucrose gradient separation. The doxorubicin localized primarily in nuclei, as expected, but appreciable amounts were also detected in mitochondria and smaller amounts in plasma membranes, microsomes, and cytoplasm. Subcellular distribution of another anticancer drug which binds to DNA, mitoxantrone, was similar. There were increased amounts of doxorubicin contained in the nuclei and all organelles of the 22:6-enriched cells. Although polyunsaturated fatty acid modification influenced the total amount of doxorubicin in fractions, the relative distribution of drug among the fractions was not different from that of the 18:1-enriched and unmodified cells. We conclude that enrichment with polyunsaturates influences total drug uptake but not proportional distribution of doxorubicin.

Doxorubicin and related anthracyclines are important antitumor drugs used to treat many human tumors. We have previously reported that enrichment of the cellular phospholipids of L1210 murine leukemia cells with polyunsaturated fatty acids results in heightened sensitivity to the cytotoxic effect of doxorubicin (1, 2). Furthermore, cellular doxorubicin accumulation is much greater by polyunsaturated-enriched cells as compared with those enriched in monounsaturates (2). However, the intracellular locus of this extra drug taken up is not known. Selective localization could explain the heightened sensitivity to the cytotoxic action of doxorubicin. In fact, little is known about the quantitative organelle-specific intracellular binding of anthracyclines. The localization of the incremental drug might also indicate the target site for the drug's action which still remains in some question (3). Since the drug must bind to its major target site to be cytotoxic, we hoped to gain insights by examining to which organelles it bound in cells whose drug sensitivity had been experimentally modified by fatty acid enrichment.

Methods. *Cell culture and fatty acid modification.* L1210 murine leukemia cells were grown in suspension culture in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% fetal bovine serum (KC Biologicals Inc., Lenexa, KS), with gentamicin sulfate (40 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2–95% air. Modification of the fatty acid composition was accomplished as previously described (1, 4). Briefly, the cells were grown for 48 hr in medium supplemented with 32 μ*M* docosahexaenoic acid (22:6) or oleic acid (18:1) (Nu Check Prep., Inc., Elysian, MN). Characterization of gradient fractions with nuclear, mitochondrial, microsomal, and plasma membranes was accomplished as previously described (5). The fatty acid composition of the fractions, has been reported (5). Briefly, the nuclei, mitochondria, plasma membranes, and microsomes of cells grown in media supplemented with 22:6 contained elevated amounts of polyunsaturated fatty acids (2.0- to 3.3-fold), especially 22:6 (22- to 43-fold), and greater mean number of double bonds (1.5- to 2.8-fold) compared to cells grown in 18:1.

Subcellular fractionation. L1210 cells were disrupted and the fractions were separated using Method I of Tsai *et al.* (6) except for the following modifications: (a) Cells suspended in homogenizing fluid were passed

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through a 25-gauge needle (30 strokes) for disruption. (b) The pellet and supernatants combined from two consecutive centrifugations of homogenate at 1300*g* for 1 min were utilized. (c) Both discontinuous gradients were made by placing 14 ml of 30% sucrose over 7 ml of 45% sucrose. (d) The nuclear fraction was further purified using 54% (2 *M*) sucrose gradients. The total fraction was taken up in 8% (0.25 *M*) sucrose and this was placed above a 54% sucrose layer and centrifuged at 58,000*g* for 30 min. Nuclei were recovered from the pellet (7). Aliquots were taken at each step of the procedure and saved for doxorubicin assays and total protein (8).

Drug localization studies. L1210 cells were washed and resuspended at 2×10^6/ml in normal saline containing 4 μM doxorubicin. Concentrations were chosen based on our previous studies (2). This concentration which is near the upper level of pharmacologic plasma levels was optimal for use of fluorescence detection of intracellular drug. Doxorubicin was extracted by placing an aliquot of each fraction into 1.5 ml acid–alcohol [concentrated H_2SO_4:propanol:water (1.5:75:23.5, v/v/v)] in an Eppendorf tapered microfuge tube and vortexing vigorously. The fluorescence of the supernatant from a 3-min 15000*g* centrifugation was determined on a Perkin–Elmer fluorescent spectrophotometer (Model 203 and Xenon lamp Model 150) with excitation at 470 nm and emission of 585 nm (2). Quinine was used as a standard to calibrate the instrument.

Studies to determine doxorubicin by a radiolabeled method were done similarly except that the cells were incubated with drug at 0.4 μM, a concentration shown to give uptake plateau at 60 min based on our previous study using radiolabelled drug (2). [^{14}C]Doxorubicin labeled at the 14-carbon position was synthesized by Research Triangle Institute (Research Triangle Park, NC) and was generously provided by the Drug Synthetic and Chemical Branch, National Cancer Institute, through arrangements by Dr. Rudge Haugwitz. The purity of the doxorubicin, which was >90%, was verified initially and at intervals thereafter, using thin-layer chromatography (9). The specific activity was adjusted to 10.6 Ci/mole in the final incubations. After 1 hr incubation, the cells were washed with ice-cold normal saline and resuspended in homogenizing medium. Subcellular fractions were generated at 4°C as described above. To assess [^{14}C]doxorubicin content, an aliquot of each fraction was placed in a scintillation vial containing 5 ml Budget-Solve scintillation fluid (RPI, Mt. Prospect, IL) and radioactivity was determined on a Beckman LS 3100 scintillation spectrometer. Differences were compared statistically using the *t* test.

Subcellular distribution studies of [^{14}C]-mitoxantrone(1,4-dihydroxy-5,8-bis[(2-((2-hydroxyethyl)amino)ethyl)amino]-9,10-anthracenedione dihydrochloride) were performed using drug kindly supplied by American Cyanamid Co. (Pearl River, NY). It was received as a powder (48 Ci/mole), constituted with distilled water and frozen at −70°C in aliquots until use. Purity was 99% as determined in our laboratory (10). The experimental time and concentration of drug used for loading the cells were chosen from our previous studies (10).

Results. *Subcellular doxorubicin distribution—fluorescence.* The distribution of drug within the various fractions was first determined by a fluorescence assay. Figure 1 shows the results for the homogenate and five major discrete fractions from 18:1- and 22:6-enriched L1210 cells. The homogenate of the 22:6-enriched cells contained 2.5-fold more drug fluorescence compared to the 18:1-enriched cells. This difference was expected from the studies of drug uptake by intact fatty acid enriched cells which showed a 1.2- to 2.3-fold increase of cell associated drug with 22:6-enriched compared to 18:1-enriched cells (2). Of the fractions, the greatest amount of doxorubicin was contained in the nuclear fraction. The doxorubicin in the nuclei of the 22:6-enriched cells was more than twofold higher than that in the 18:1-enriched cells. Although not statistically significantly different, the drug contained in the other fractions was higher in the 22:6-enriched cells in each case except the cytosol which contained only trace amounts of doxorubicin in either cell type.

Studies of doxorubicin distribution in intracellular sites were also carried out on unmodified cells. The fatty acid composition of these cells is similar to those grown in 18:1-

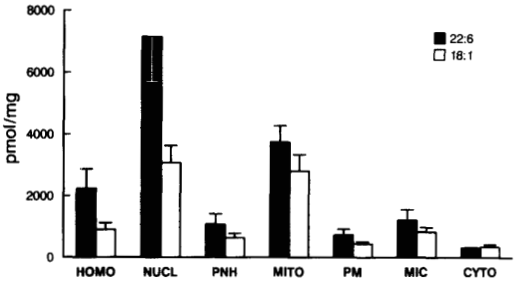

FIG. 1. Subcellular doxorubicin distribution. L1210 cells were incubated in medium containing 4 μM doxorubicin. The washed chilled cells were ruptured and the subcellular fractions were separated. The fractions were placed in acid–alcohol and centrifuged, and the supernatants were read in a fluorescent spectrophotometer. Shown are the means ± SE in pmole/mg protein of five to six separate determinations. Of the fractions separated from the homogenate there was a significantly different value of 22:6- vs 18:1-enriched cells only for the nuclei ($P < 0.05$). HOMO, homogenate; NUCL, nucleus; PNH, post-nuclear homogenate; MITO, mitochondria; PM, plasma membrane; MIC, microsomes; CYTO, cytoplasm.

supplemented medium [4]. The relative distribution of drug into the various fractions was similar to the fatty acid modified cells. For example, doxorubicin expressed as pmole/mg protein was four- to fivefold greater in nuclei than in post-nuclear homogenate. Likewise, the drug in the mitochondria fraction was twofold greater than either plasma membrane or microsome fractions and only small amounts were detected in cytoplasm.

Subcellular doxorubicin distribution—Radiolabeled drug. In planning these studies, there was some concern that there might be loss of drug from subcellular fractions during the washing procedure. Therefore, we repeated the studies, determined the amount of doxorubicin in each wash, and added that amount to the value obtained for the corresponding fraction. In order to perform this type of study, radiolabeled drug was used to allow detection of small amounts of drug in the wash media. The results of these studies are shown in Table I. When the results are expressed as percentage distribution of recovered drug, there was no difference in the 22:6- versus 18:1-enriched cells. The only difference which was statistically significant was the proportion of drug in the microsome fraction. We doubt that it is of much biologic importance since there is only a small amount of drug in this fraction and the difference between 22:6- and 18:1-enriched cells is relatively small. In Table I the content is expressed as percentage drug recovered per fraction and shows that more total doxorubicin was contained in the post-nuclear homogenate fraction (75%) than in the nuclear fraction (25%). However, when values were corrected for amount of protein recovered from each fraction, the nuclear fraction showed the greatest enrichment of drugs (Fig. 1).

Subcellular mitoxantrone distribution. Because of the paucity of studies in the literature on subcellular drug distribution performed on enzyme-characterized gradient-separated fractions, we studied the distribution of another DNA-binding drug, mitoxantrone, in order to compare drug distribution and recovery with that of doxorubicin. [^{14}C]Mitoxantrone was incubated with unmodified L1210 cells and fractions were separated as with doxorubicin. When expressed as recovered drug per milligram of organelle protein, the greatest amount of mi-

TABLE I. RELATIVE SUBCELLULAR DISTRIBUTION OF DOXORUBICIN

Fraction enriched for	22:6	18:1
Distribution in homogenate[a]		
Post-nuclear homogenate	73.2 ± 5.6	76.4 ± 5.6
Nuclei	26.8 ± 5.6	23.6 ± 5.6
Distribution in nuclei-free homogenate[b]		
Mitochondria	51.6 ± 14.1	47.3 ± 1.4
Plasma membrane	30.8 ± 16.5	20.7 ± 2.0
Microsomes	10.7 ± 1.4	26.0 ± 0.9
Cytosol	6.9 ± 1.3	6.7 ± 2.4

Note. L1210 cells were incubated with [^{14}C]doxorubicin (0.4 μM, 10.6 Ci/mole) for 1 hr and washed with ice-cold media. The cells were disrupted and the fractions were separated using sucrose gradients. Radioactivity was determined in each fraction and to this was added the radioactivity in the two washes of each fraction. Shown are values expressed as a percentage of recovered radioactivity. The values are the means and SE of three separate determinations.

[a] Percentage calculated on basis of total radioactivity recovered from homogenate.

[b] Percentage calculated on basis of total radioactivity recovered from sucrose gradient.

toxantrone was recovered in the nuclear fraction (Table II). This distribution is similar to that of doxorubicin (Fig. 1). From these studies, we conclude that the general patterns of distribution and recovery of mitoxantrone and doxorubicin are similar.

Discussion. The data on the subcellular location of this important anthracycline anticancer drug indicate that polyunsaturated fatty acid enrichment increases the amount of doxorubicin contained in the nuclei. Although only the nuclei contained statistically more doxorubicin, every particulate fraction of the 22:6-enriched cells was numerically higher in drug content than the corresponding fraction of the 18:1-enriched cells. The higher absolute levels of accumulated drug in the nuclear fraction of the 22:6-enriched cells allows for more biochemical and physical interaction at the target site and likely leads to the heightened cytotoxic susceptibility (1, 2). However, when the percentage distribution of the drug was examined, none of the fractions of the 22:6-enriched cells accumulated more drug than the corresponding fraction of the 18:1-enriched cells.

The other important finding of this study relates to the subcellular localization of doxorubicin per se. The majority of the drug expressed per milligram of protein was contained in the nucleus and mitochondria with small amounts in plasma membrane, microsomes, and cytosol, regardless of fatty acid composition. We recognize that disruption of the cells may lead to redistribution of drug. We did see some evidence of recovery anomalies in sucrose gradient fractions (vida infra). Our observations should be interpreted with these possible limitations in mind.

TABLE II. SUBCELLULAR DISTRIBUTION OF MITOXANTRONE

Fraction	Mitoxantrone concentration	
	pmole/mg	pmole/10^8 cells
Homogenate	63 ± 17	595 ± 179
Post-nuclear homogenate	57 ± 13	501 ± 138
Nuclei	117 ± 22	16 ± 3
Mitochondria	86 ± 36	37 ± 15
Plasma membrane	50 ± 11	6 ± 2
Microsomes	50 ± 13	5 ± 1
Cytosol	15 ± 2	68 ± 5

Note. L1210 cells (unmodified) were incubated with [^{14}C]mitoxantrone (4 μM, 48 Ci/mole) for 30 min and washed with ice-cold media. The cells were disrupted and the fractions were separated as described in text and Table I. Values are means and SE of three separate determinations.

Previous studies have reported certain aspects of intracellular drug localization. Investigators using fluorescence microscopy have demonstrated that doxorubicin localizes primarily in cell nuclei of cultured L1210 cells (11), human lymphoblasts and fibroblasts (12), and normal cells (13). Small amounts were detectable in cytoplasm of liver and kidney. However, quantification of fluorescence in various intracellular locations was not possible using the cytofluorescent technique, nor is it possible to distinguish drug associated with particular cytoplasmic organelles. Johnson *et al.* (14), using radiolabeled drug, compared doxorubicin uptake into two fractions by separating lysates into pellet and supernatant fractions using a 1000*g* for 15 min centrifugation. In these L5178Y lymphoblasts, greater than 75% of the drug was localized in the pellet which was not characterized biochemically. Most studies of nuclear binding of anthracyclines using different detection techniques have been done by incubating drug with isolated nuclei (15, 16).

The 18:1-enriched cells serve as a control cell for comparison with the polyunsaturated-rich cells. We have previously demonstrated that cells grown in media enriched in 18:1 are similar in fatty acid composition and physical properties to unmodified cells (4). We avoided the use of unmodified cells in any detail in the current study because such a comparison of modified and unmodified cells would not take into account the fact that only one of the cell types would have been exposed to exogenous supplemented fatty acid in the media. However, for the observations on subcellular distribution they represent a reasonable approximation to unmodified cells since they are similar in fatty acid composition (4), cholesterol and phospholipid content (4), membrane order (4), culture doubling time (1), cloning efficiency (1), and doxorubicin sensitivity (1).

One limitation of our approach concerned

doxorubicin recovery. The [^{14}C]doxorubicin studies of Table I in which the wash media was taken into account were carried out to minimize unaccounted-for drug. After the initial separation of homogenate into nuclei and post-nuclear fractions, about 80% of the drug was recovered. This indicates that our conclusion regarding the nuclei as a major site of intracellular distribution of doxorubicin of Fig. 1 expressed as drug per milligram protein can be reached with high confidence. However, of the total drug contained in the post-nuclear homogenate, only 32% was usually recovered in subsequent fractions from sucrose gradients. This should not be confused with the values of Table I which express percentage distribution of recovered drug. The gradient separations were carried out as quickly as possible and the temperature was kept low in order to minimize loss of drug. However, in spite of these efforts, only about one-third of the drug in the post-nuclear fraction could be accounted for. The recovery of mitoxantrone was also similar in that there was satisfactory accounting for the drug after the initial separation into nuclear and post-nuclear fractions (87%), but poor recovery of the gradient fractionation of the post-nuclear fraction (23%). If there was selective loss of drug or if the drug redistributed into fractions which did not contain it in the intact cell, then the results regarding the drug in the separate fractions of the post-nuclear homogenate could be misleading.

In our study which allows quantification of drug in fractions enriched for various organelles, we found considerable drug localized with the mitochondria marker. This doxorubicin may be bound to mitochondrial membranes. Doxorubicin binds preferentially to cardiolipin which is found predominantly in mitochondrial membranes (17–19). Averbuch *et al.* (20) used a radiolabeled photoactive anthracycline analogue to demonstrate a low molecular weight polypeptide which binds doxorubicin in the mitochondria of P388 leukemia cells. However, in those studies homogenates rather than intact cells were incubated with drug. It is possible that the physical effects which the drug is known to exert on plasma membranes may also pertain to some extent to this organelle.

There is considerable evidence that doxorubicin has a pharmacologic effect on plasma membranes [3]. Yee *et al.* (21) used photoaffinity labeling to demonstrate that daunorubicin, a related anthracycline, photoincorporated into cell surface protein but not DNA. This drug localizing to plasma membrane may explain the known effect of doxorubicin on fluidity (22), lectin-mediated cell agglutination (22, 23), liposome fusion rate (19), and prostanoid production (24). Our study indicates that 15–20% of the recovered drug localized in and presumably bound to the membrane fractions enriched for 5′-nucleotidase, the plasma membrane marker. Although this is a small amount compared with that contained in nuclei, it would appear to be enough to exert a physical or chemical effect on a sensitive target. That some appreciable amount of drug is found in the fraction enriched in 5′-nucleotidase supports the possibility that plasma membranes could be an important target of the drug's cytotoxic action.

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