Transport Mechanism of Anthracycline Derivatives in Rat Polymorphonuclear Leukocytes: Effect of Sodium Fluoride on Pirarubicin Uptake

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We previously revealed that pirarubicin (THP) was actively taken up by rat polymorphonuclear leukocytes *via* a carrier-mediated transport system. In the experiment on the effects of the metabolic inhibitors, rotenone, 2,4-dinitrophenol and sodium cyanide significantly decreased the THP transport. However, sodium fluoride (NaF) significantly increased the uptake, and this result is different from that in some reports. Therefore, we examined the action of NaF on THP uptake by the leukocytes to clarify the discrepancy in the effect of NaF on drug transport. The accelerating effect of 30 mM NaF on the THP uptake by the cells had an optimum period of action (15–20 min), and was concentration-dependent (5–30 mM). Thirty mM potassium fluoride, as well as NaF, increased the uptake amount. On the other hand, NaF (5–30 mM) dose-dependently decreased the ATP content in these cells. Additionally, the viable cells in the reaction suspension decreased by about 40% after incubation with 30 mM NaF for 15 min. Observing these leukocytes treated with NaF by optical microscopy, swelling of the cell and an alteration of the nuclei form occurred. On the basis of these results, we speculated that the increased THP transport in polymorphonuclear leukocytes by NaF, probably F^- , might be due, at least in part, to an alteration of the morphological form.

Key words polymorphonuclear leukocyte; increased accumulation; sodium fluoride; pirarubicin transport

Previously, we reported that pirarubicin (THP) was actively taken up by PMN cells *via* a carrier-mediated transport system,¹⁾ and furthermore, this mechanism would be common to that in daunorubicin (DNR) and doxorubicin (ADR).²⁾ In the experiment using the metabolic inhibitors, 10 μ M rotenone, 4 mM 2,4-dinitrophenol and 2 mM sodium cyanide significantly reduced THP transport in PMN cells. However, sodium fluoride (NaF), which has generally been known to deplete cellular ATP, significantly increased the uptake amount of THP, contrary to the report of Markes *et al.*, in which NaF markedly decreased the benzomorphan transport in rat leukocytes due to the depletion of cellular ATP.³⁾

Herein, we performed further experiments to clarify the effect of NaF on THP transport in PMN cells.

MATERIALS AND METHODS

Chemicals Pure THP, tetrahydropyranyldoxorubicinol, the internal standard for HPLC analysis, and DNR were obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), and ADR was provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). All other reagents were obtained commercially and were of analytical grade, requiring no further purification.

Animals Male Wistar strain rats (Japan SLC, Inc., Hamamatsu, Japan), weighing 200–300 g, were used in the experiments.

Preparation of PMN Cells 0.1% gel-Hanks solution (gel-HBSS) (pH 7.4) was used in this study as the incubation medium.

PMN cells were prepared from rats using the same procedure described previously.¹⁾ The cells obtained were subjected to total and differential counts using an improved

Neubauer counting chamber (Clay Adams). The PMN cells were stained with gentiana violet stain and counted as polymorphonuclear cells under a light microscope. If 98% or more of the cells showed a normal function in the Trypan blue exclusion test to determine the cell viability,⁴⁾ the cells were used in the experiments.

Uptake Experiments In the experiments for THP uptake, preincubation of the reaction suspension (cell density; 5×10^6 cells/ml) was performed for 30 min at 37 °C with shaking (160 strokes/min). The reaction was initiated by the addition of the drug solution dissolved in distilled water to the incubated medium (the final THP concentration in the medium was 0.3 μ M), and was terminated by centrifugation of the reaction suspension at appropriate time intervals as reported previously.¹⁾

Assay Procedure of THP The THP concentrations in PMN cells were determined by HPLC as reported previously.^{5,6)}

Assay Procedure to Determine Cellular ATP Content The ATP content in viable PMN cells was determined by HPLC as described below.⁷⁾ The reaction suspension containing PMN cells was incubated for 15 min after the addition of NaF (0, 5, 15 or 30 mM). Thereafter, the cells were centrifuged at $450 \times g$ for 5 min, washed three times with saline, and centrifuged at $10000 \times g$ for 10 s again after the addition of 1 M $HClO_4$ and vortex. The supernatant was filtered with a membrane filter (pore size; 0.45 μ m, Gilson). A 50 μ l portion of the filtrate obtained by two-fold dilution with water was injected into the HPLC apparatus. The HPLC system consisted of a Shimadzu LC-6A equipped with a UV spectrophotometric detector (Shimadzu, SPD-6A). The analytical conditions were as follows: The column was a STR-ODS II (5 μ m, 250 mm $\times$ 4 mm i.d., Shimadzu), with a mobile phase

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consisting of 20 mM citric acid containing 30 mM diethylaminoethanol and a flow rate of 1.5 ml/min. The absorbance was measured at 260 nm. No interference peaks due to endogenous substances were observed.

Protein Assay After the incubation of PMN cells with 30 mM NaF for 0, 15, 30 or 60 min, the cells were centrifuged at $450 \times g$ for 5 min and washed three times. The protein content in the pellet, which consisted of viable cells, was determined by the method of Lowry *et al.*⁸⁾

Statistical Analysis The data are expressed as the mean $\pm$ S.E., and the significance was evaluated by the Student's *t*-test or ANOVA as appropriate, and was defined as a *p* value of 0.05 or less.

RESULTS AND DISCUSSION

Figure 1 shows the effect of NaF on the THP uptake by PMN cells. In all groups, it was thought that THP was concentrated into the PMN cells, because the THP concentration in the PMN cells (5×10^6 cells) at equilibrium was calculated to be approximately 76 nmol/ml, and this level was much higher than that in the extracellular fraction (0.0276 nmol/ml), assuming the diameter of a PMN cell was 10 μ m. In addition, we reported that about 27% of the apparent accumulation amount of THP in the PMN cells was bound to the cell membrane surface.¹⁾ Therefore, it appeared that THP was taken up by the PMN cells against the concentration gradient.

As shown in Fig. 1, in the pretreatment group, in which 30 mM NaF was added to the reaction suspension at 15 min before the THP addition, the amount of THP uptake significantly increased up to 5 min compared with that in the control group. Thereafter, it was reduced in a time-dependent manner, and significantly ($p < 0.05$) decreased compared with that in the control group at 15 min, because of the extensive reduction of viable cells (Fig. 5). In the simultaneous treatment group (30 mM NaF and THP were added to the reaction suspension simul-

taneously at time zero), although NaF did not affect the THP uptake in the initial phase up to 10 min, the THP incorporation into PMN cells in this group tended to increase compared with that in the control group at 15 min. So it was found that NaF increased the THP uptake by PMN cells at 15 to 20 min after its addition, suggesting that this effect of 30 mM NaF had an optimum period of action. Furthermore, the uptake of ADR and DNR was significantly increased by 30 mM NaF under the same condition (data not shown). However, it is not yet clear from this study whether the effect of NaF is specific to the uptake of these anthracycline derivatives. Thus, in the succeeding experiments, THP uptake was assessed after the pretreatment of PMN cells with NaF for 15 min.

Figure 2 shows the effect of the additional amount of NaF on THP uptake. It was observed that 5 mM of NaF did not affect the transport, but 15 and 30 mM additions significantly increased the THP uptake, by about 60%, indicating that the accelerating effect of NaF on THP uptake had a concentration dependency.

Fig. 2. Effect of NaF Concentration on THP Uptake by PMN Cells

Each medium was preincubated with 0, 5, 15 or 30 mM of NaF for 15 min at 37°C, and then incubated with 0.3 μ M of THP for 1 min at 37°C. Each column represents the mean $\pm$ S.E. of three experiments. *a)* $p < 0.01$ significantly different from the control value.

Fig. 1. Time Courses of THP Uptake by PMN Cells Treated with NaF

Squares, triangles and circles represent, respectively, the control group of cells incubated with 0.3 μ M of THP alone, the simultaneous treatment group which was incubated with both 0.3 μ M of THP and 30 mM of NaF, and the pretreatment group which was preincubated with 30 mM of NaF for 15 min and then incubated with 0.3 μ M of THP for the indicated time at 37°C. Each point represents the mean $\pm$ S.E. of three experiments. *a)* and *b)* $p < 0.05$ and 0.01 significantly different from the control group, respectively. *c)* and *d)* $p < 0.05$ and 0.01 significantly different from the simultaneous treatment group, respectively.

Fig. 3. Effects of NaF and KF on THP Uptake by PMN Cells

Each medium was preincubated with or without 30 mM of either NaF or KF for 15 min, and then incubated with 0.3 μ M of THP for 1 min at 37°C. Each column represents the mean $\pm$ S.E. of three experiments. *a)* $p < 0.001$ significantly different from the value of control.

Akerboom *et al.*⁹⁾ reported that NaF stimulated the transport of dinitrophenyl glutathione in inside-out plasma membrane vesicles prepared from human erythrocytes. They speculated that this was due to the direct interaction of the fluoride ion (F^-) with this transporter. Therefore, we performed the experiment using potassium fluoride (KF) to confirm whether or not either the sodium ion or F^- were involved individually in the increase of THP uptake (Fig. 3). The uptake in both the NaF and KF group significantly increased compared with that in the control group, and the increased level of THP accumulation by KF was approximately equal to that by NaF. This suggested that the increase in THP transport by NaF might be due to F^- .

On the other hand, Marks and Medzihradsky *et al.* reported that incubation with 30 mM NaF for 60 min decreased the uptake amounts of the drug by rat leukocytes.^{3,10,11)} These decreases correlated with those of the cellular ATP content.³⁾ This action of NaF did not show competitive inhibition,¹²⁾ and there was no effect

of incubation with 3 and 6 mM NaF for 60 min on the number and viability of these cells.¹¹⁾ Therefore, at first we tried to determine the ATP content in the viable PMN cells incubated with NaF. As shown in Fig. 4, NaF apparently reduced the ATP content in PMN cells, and the extent of ATP decrease depended on the NaF concentration in the medium, similar to the result reported by Marks and Medzihradsky.³⁾ In our previous report, the THP transport in PMN cells appeared to depend on energy.¹⁾ Therefore, the uptake was diminished if the ATP content in the cells decreased. However, the THP uptake was conversely increased by the addition of NaF as described above. Consequently, it was conceivable that NaF increased THP uptake *via* an energy-independent transport system; for example, as the result of any changes in the structure and integrity of the cell membrane, the membrane permeability, *etc.*

Next, we verified the viability of PMN cells treated with 30 mM NaF using the Trypan blue exclusion test.⁴⁾ As Fig.

Fig. 4. Effect of NaF on ATP Content in Viable PMN Cells

Each medium was preincubated with 0, 5, 15 or 30 mM of NaF for 15 min, and thereafter, the cellular ATP content was determined. Each column represents the mean $\pm$ S.E. of three experiments. a) $p < 0.001$ significantly different from the value of control.

Fig. 5. Effect of NaF on Viable PMN Cell Count in Reaction Suspension

Each medium was incubated with 30 mM of NaF for the indicated time at 37°C, and then the Trypan blue exclusion test was performed. Each column represents the mean $\pm$ S.E. of three experiments. a) and b) $p < 0.01$ and 0.001 significantly different from the value of 0 min, respectively.

Fig. 6. Microscopic Photographs ($\times 400$) of PMN Cells Treated with 30 mM of NaF

PMN cells were treated with or without 30 mM of NaF for 15 min at 37°C, then the cells were stained by gentiana violet dye. A, control; B, NaF 30 mM.

5 shows, 30 mM NaF decreased the viable cells in the reaction suspension time-dependently, and there was about a 40% decrease after incubation with 30 mM NaF for 15 min. However, the THP uptake increased under this condition.

From these results, we were lead to the hypothesis that NaF might not kill, but only alter the structure of PMN cells themselves under our experimental conditions. Therefore, we tried to confirm their morphological aspects using light microscopy after staining with a gentiana violet dye (Fig. 6). In the control group, the plasma-membrane and nuclei of the PMN cells were normal and there was no alteration. In contrast, a marked morphological alteration, *e.g.*, swelling of the whole cell and alteration of the nuclei form, was observed in the NaF-treated cells, which were not stained by trypan blue dye. These findings suggested that the PMN cells were not dead but were changed markedly in some function by NaF.

Accordingly, it was speculated that the increment of THP uptake in PMN cells following treatment with NaF might be attributed, at least in part, to the alteration of their morphological form by F^- . Additional studies are in progress to clarify this detailed mechanism.

REFERENCES

- 1) K. Nagasawa, M. Nomiyama, N. Ohnishi, T. Yokoyama, S. Iwakawa, K. Okumura, *Biol. Pharm. Bull.*, **17**, 696 (1994).
- 2) K. Nagasawa, M. Nomiyama, N. Ohnishi, T. Yokoyama, S. Iwakawa, K. Okumura, *Biol. Pharm. Bull.*, **17**, 1305 (1994).
- 3) M. J. Marks, F. Medzihradsky, *Mol. Pharmacol.*, **10**, 837 (1974).
- 4) E. A. Boyse, L. J. Old, I. Chouroulinkov, *Methods Med. Res.*, **10**, 39 (1964).
- 5) K. Nagasawa, T. Yokoyama, N. Ohnishi, S. Iwakawa, K. Okumura, Y. Kosaka, K. Sano, R. Murakami, H. Nakamura, *J. Pharmacobio-Dyn.*, **14**, 222 (1991).
- 6) K. Nagasawa, N. Kitada, C. Tsuji, M. Ogawa, T. Yokoyama, N. Ohnishi, S. Iwakawa, K. Okumura, *Chem. Pharm. Bull.*, **40**, 2866 (1992).
- 7) Shimadzu Application News, No. L207 (1992).
- 8) O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 9) T. P. M. Akerboom, G. Bartosz, H. Sies, *Biochim. Biophys. Acta*, **1103**, 115 (1992).
- 10) F. Medzihradsky, M. J. Marks, E. A. Carr, *Biochem. Pharmacol.*, **21**, 1625 (1972).
- 11) M. J. Marks, F. Medzihradsky, *Biochem. Pharmacol.*, **23**, 2951 (1974).
- 12) F. Medzihradsky, M. J. Marks, J. I. Metcalfe, *Adv. Biochem. Psychopharmac.*, **8**, 537 (1974).