# TUMOR CELL PERMEABILITY TO PEPLOMYCIN

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The uptake of [$^3$H]peplomycin-Cu(II) ([$^3$H]PEP-Cu(II)) into various tumor cell lines was studied. The time course of [$^3$H]PEP-Cu(II) uptake into AH66, AH66F, Ehrlich and P388 cells was biphasic. The first phase of uptake was completed within 5 minutes. The second, slower phase, of uptake into AH66, AH66F and Ehrlich cells increased linearly with incubation time, but that into P388 cells reached a plateau level. In L1210 cells, only the first rapid uptake was observed. The lower uptake into P388 and L1210 cells during the second phase may be related to their insensitivity to PEP.

However, the uptake into AH66F cells was higher than that into AH66 cells, although AH66F cells were less sensitive to PEP than AH66 cells. Deamide PEP was detected in intact cells which had taken up [$^3$H]PEP-Cu(II) during 4 hours. This confirmed that PEP-Cu(II) was transported into the cell, the copper removed and PEP metabolized to deamide PEP.

[$^3$H]PEP-Cu(II) uptake into AH66 and AH66F cells increased in proportion to the extracellular concentration of drug up to at least 200 $\mu$g/ml, suggesting that uptake was not mediated by a carrier system. Metabolic inhibitors such as $NaN_3$ and 2,4-dinitrophenol enhanced [$^3$H]PEP-Cu(II) uptake, but did not influence efflux. Uptake was also enhanced by membrane modifiers such as dibucaine and chlorpromazine which increase the fluidity of lipid membranes. The results suggest that PEP-Cu(II) was taken up into tumor cells by passive diffusion, controlled by an energy-dependent cell membrane barrier.

Bleomycins (BLMs), a group of glycopeptide antibiotics, are used in the treatment of human cancers. While BLM produces only minor bone marrow toxicity, its clinical usefulness is limited by pulmonary toxicity. Peplomycin (PEP) is a biosynthetic BLM derivative exhibiting lower pulmonary toxicity and stronger antitumor activity compared to BLM[1,2]. The primary target site of this drug is generally accepted to be nuclear DNA, because it efficiently degrades isolated DNA in the presence of Fe(II) and molecular oxygen, and inhibits intracellular DNA synthesis[3,4]. The transport process into tumor cells is clearly essential for its antitumor activity. Since this process may be one of the factors determining the sensitivity of tumor cells to PEP, we investigated the PEP permeability of various sensitive or insensitive tumor cells and its mode of transport.

BLM chelates cupric ion and when copper-free BLM is administered to animals, it chelates cupric ion in the blood[5]. Since the BLM-copper complex inhibits the growth of tumor cells *in vitro*[6], this complex is thought to be the main form acting on tumors *in vivo*. Therefore, we used the PEP-copper complex (PEP-Cu(II)), and not copper-free PEP, for the present study.

## Materials and Methods

### Materials

PEP sulfate, deamide PEP and [$^3$H]PEP-Cu(II) were prepared at Nippon Kayaku Co., Ltd., Japan. [$^3$H]PEP-Cu(II) was purified using a Lober column LiChroprep Si 60 (10×240 mm) (E. Merck, West Germany) before use. Five hundred $\mu$l of [$^3$H]PEP-Cu(II) (5 mg) was applied on this column and

eluted with a solvent consisting of $CH_3OH$ - $CH_3CN$ - 20% $CH_3COONH_4$ - $CH_3COOH$ (560 : 440 : 100 : 0.5). Absorbance was monitored at 292 nm and the [$^3$H]PEP-Cu(II) fraction was collected, desalted and lyophilized. Sodium azide and 2,4-dinitrophenol (DNP) were purchased from E. Merck, West Germany and Tokyo Kasei Kogyo Co., Ltd., Japan, respectively. Dibucaine hydrochloride, chlorpromazine hydrochloride, vinblastine sulfate and colchicine were purchased from Sigma Chemical Company, U.S.A.

Cells

AH66 and AH66F hepatoma cells were maintained by intraperitoneal passage of ascites in Donryu rats. P388 and L1210 leukemia cells were maintained by intraperitoneal passage of ascites in DBA/2 mice. Ehrlich cells were maintained by intraperitoneal passage of ascites in ICR mice, and cells were obtained from the peritoneal cavity of rats or mice on day-5 or 6 after inoculation. The medium used in culture was MEM for suspension culture (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 20 mM HEPES buffer and 10% calf serum (Flow Laboratories Inc., U.S.A.) for AH66, AH66F and Ehrlich cells, and RPMI 1640 (Gibco Laboratories Inc., U.S.A.) supplemented with 20 mM HEPES buffer and 10% fetal calf serum (Flow Laboratories Inc., U.S.A.) for P388 and L1210 cells.

Growth Inhibitory Activity of PEP and PEP-Cu(II)

AH66 and AH66F cells were cultured at an initial density of $2\times10^4$ cells/ml/well. After preincubation at 37°C for 2 hours in a $CO_2$ incubator, drugs were added and the cultures were incubated a further 48 hours. Inhibition of growth was estimated from cell numbers before and after treatment with drugs.

[$^3$H]PEP-Cu(II) Uptake into Various Tumor Cells

Tumor cells were suspended in fresh medium at a concentration of $2\times10^7$ cells/ml. Then, [$^3$H]-PEP-Cu(II) was added and the cells were incubated with shaking for the indicated times at 37°C. After incubation, 0.5 ml of the cell suspension was centrifuged with 5 ml of ice-cold phosphate-buffered saline (PBS), and washed twice with ice-cold PBS and centrifugation (2,000 rpm, 30 seconds). The cells were solubilized with 0.75 ml of 1 N NaOH for 1 hour at 60°C, and neutralized with 0.20 ml of 5 N HCl. Radioactivity was determined in 5 ml of ACS-II liquid scintillation cocktail (Amersham Co., Canada).

Intracellular Degradation of [$^3$H]PEP-Cu(II)

Cells were incubated with [$^3$H]PEP-Cu(II) for the indicated times at 37°C. After the cells were washed with ice-cold PBS and centrifuged, they were treated with 1 ml of 5% TCA containing 10 $\mu$g of non-radioactive PEP-Cu(II) and 10 $\mu$g of deamide PEP-Cu(II). The solution was ultrasonicated, centrifuged at 3,000 rpm for 10 minutes and the supernatant neutralized with 3.2 N KOH. $CuCO_3\cdot Cu(OH)_2\cdot H_2O$ was added and the solution applied to a $C_{18}$SEP-PAK cartridge (Waters Associates Inc., U.S.A.) and eluted with $CH_3OH$ - 0.005 N HCl (1 : 1). The eluate was neutralized with 1/15 M phosphate buffer (pH 7.2) and lyophilized. The sample was dissolved in $H_2O$ and analyzed by high pressure liquid chromatography (HPLC).

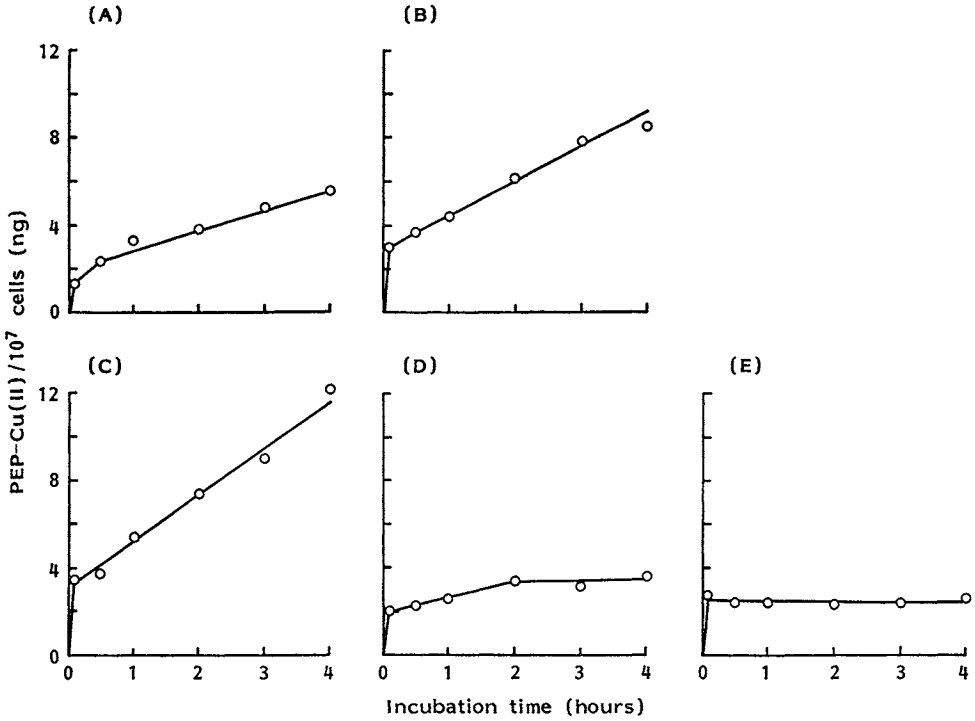
## Results

Fig. 1 shows the uptake of [$^3$H]PEP-Cu(II) by five tumor cell lines, AH66, AH66F, Ehrlich, P388 and L1210 cells. Except for L1210 cells, the time course of [$^3$H]PEP-Cu(II) uptake was biphasic. The first phase was completed within 5 minutes. In the second phase, the uptake into AH66, AH66F and Ehrlich cells increased linearly with incubation time. The second slow uptake into P388 cells was not linear but reached a plateau level. In L1210 cells, only the first rapid uptake was observed. The first phase uptake was greatest with Ehrlich cells and least with P388 cells, with a 2-fold difference between them. In contrast, the second phase uptakes were very different among tumor cell lines. The cell: medium distribution ratio of the drug after a 4-hour incubation with Ehrlich cells was 0.0677.

AH66 cells were much more sensitive to PEP than AH66F cells. As Table 1 shows, the $IC_{50}$ of

Fig. 1. Time course of [$^3$H]PEP-Cu(II) uptake into AH66 (A), AH66F (B), Ehrlich (C), P388 (D) and L1210 (E) cells.

The uptake was measured by incubating cells with 4 $\mu$g/ml of [$^3$H]PEP-Cu(II).




PEP-Cu(II) against AH66 cells was 1/93 of that against AH66F cells. The growth inhibitory activities of PEP-Cu(II) against the two cell lines were almost identical to those of copper-free PEP. Hence we compared [$^3$H]PEP-Cu(II) uptake into AH66 cells with that into AH66F cells. The dose-response of [$^3$H]PEP-Cu(II) uptake into AH66 and AH66F cells is shown in Fig. 2. The uptake into AH66F cells was higher than that into AH66 cells. The uptake into the two tumor cell lines increased in proportion to the extracellular concentration of drug up to 200 $\mu$g/ml. Linearity was observed during 5 minutes, 1 and 4 hours of incubation. This suggests that the uptake of [$^3$H]PEP-Cu(II) is by passive diffusion, rather than mediated by a carrier system.

Table 1. Growth inhibition of AH66 and AH66F cells by PEP-Cu(II) and PEP.

| Drug | $IC_{50}$ ($\mu$g/ml) | |
|---|---|---|
| | AH66 | AH66F |
| PEP-Cu(II) | 0.028 | 2.6 |
| PEP | 0.028 | 3.6 |

The time course of the efflux of [$^3$H]PEP-Cu(II) from AH66 and AH66F cells is shown in Fig. 3. Tumor cells were incubated with [$^3$H]PEP-Cu(II) for 5 minutes or 4 hours, and washed with ice-cold PBS five times in order to remove extracellular [$^3$H]PEP-Cu(II). The washed tumor cells were further incubated for the indicated time, and the remaining radioactivity in the tumor cells was measured. The time course of efflux after 4 hours uptake of [$^3$H]PEP-Cu(II) was biphasic. On the other hand, the efflux after 5 minutes uptake was almost completed within 15 minutes. The first phase of efflux after 4 hours uptake probably corresponds to the efflux after 5 minutes uptake. This result indicates that [$^3$H]PEP-Cu(II) taken up by tumor cells exists in two states.

Fig. 2. Dose-response of [$^3$H]PEP-Cu(II) uptake into AH66 (A) and AH66F (B) cells. Incubation time was 5 minutes (○), 1 hour (△) and 4 hours (□).

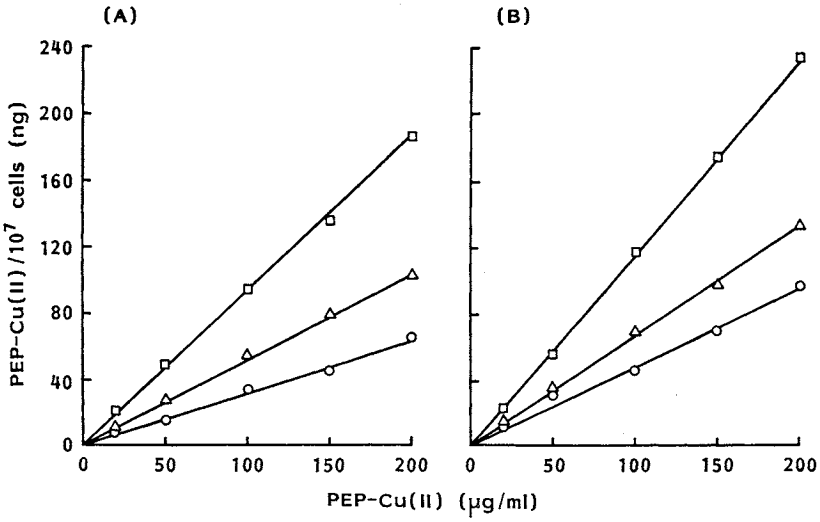
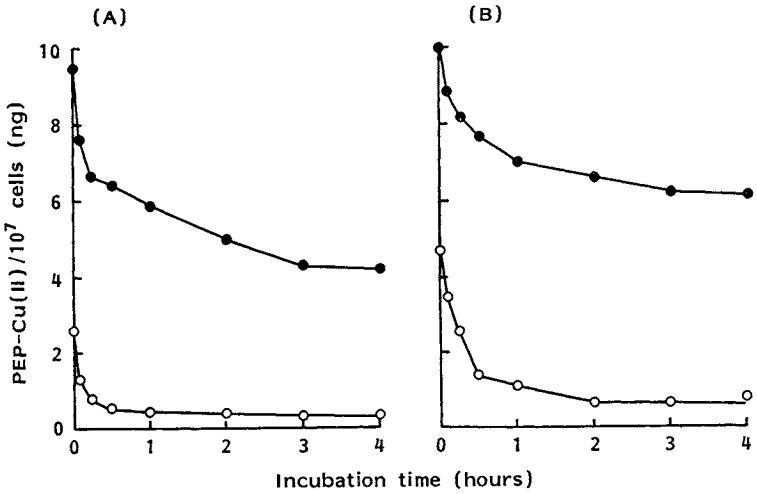



Fig. 3. Time course of efflux of [$^3$H]PEP-Cu(II) from AH66 (A) and AH66F (B) cells.

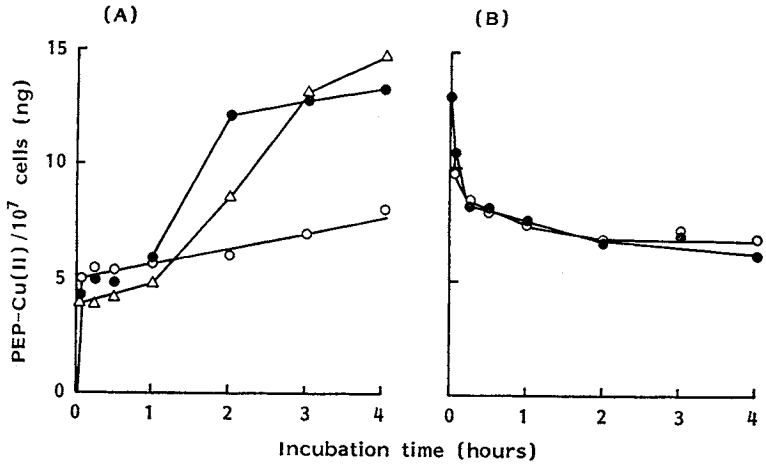



Tumor cells were incubated with 4 μg/ml of [$^3$H]PEP-Cu(II) for 5 minutes (○) or 4 hours (●) and washed with ice-cold PBS five times. The washed tumor cells were further incubated and the radioactivity remaining on the tumor cells was determined.

To determine whether the uptake of [$^3$H]PEP-Cu(II) was dependent upon energy produced by the mitochondorial transport system, cells were preincubated with either 10 mM $NaN_3$ or 1 mM DNP for 10 minutes at 37°C. These metabolic inhibitors enhanced rather than inhibited [$^3$H]PEP-Cu(II) uptake into AH66F cells (Fig. 4). However, the first rapid uptake was not influenced by these inhibitors. The effect of 1 mM DNP on efflux was also examined. DNP had little influence on the efflux of [$^3$H]PEP-Cu(II) from AH66F cells (Fig. 4B).

The effect of cell membrane modifiers such as dibucaine and chlorpromazine on [$^3$H]PEP-Cu(II)

Fig. 4. Effect of metabolic inhibitors on [$^3$H]PEP-Cu(II) uptake (A) and efflux (B) in AH66F cells.

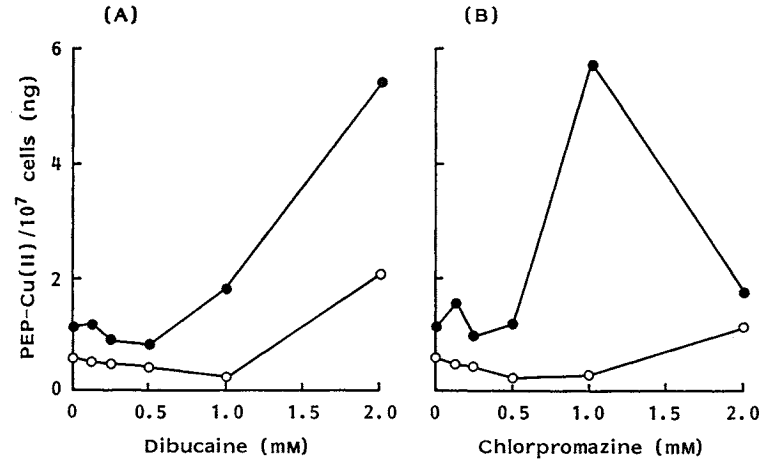



(A) After AH66F cells were preincubated with 1 mM DNP (●), 10 mM $NaN_3$ (△) or not (○), 4 μg/ml of [$^3$H]PEP-Cu(II) was added and the uptake was determined.

(B) After AH66F cells were incubated with 4 μg/ml of [$^3$H]PEP-Cu(II) for 3 hours, the cells were washed and further incubated with (●) or without (○) 1 mM DNP. The efflux was determined as described in Fig. 3.

Fig. 5. Effect of dibucaine (A) and chlorpromazine (B) on [$^3$H]PEP-Cu(II) uptake into AH66F.

The uptake was measured by incubating cells with 0.5 μg/ml of [$^3$H]PEP-Cu(II) for 5 minutes (○) or 4 hours (●).




uptake into AH66F cells is shown in Fig. 5. These agents are reported to interact extensively with the cell membrane[7]. At concentrations of 1 mM or above, these agents enhanced [$^3$H]PEP-Cu(II) uptake. This enhancement was more pronounced in 4 hours uptake than 5 minutes uptake. The effect of inhibitors of tubulin polymerization such as vinblastin and colchicin on [$^3$H]PEP-Cu(II) uptake into AH66F cells was also examined. At 1 mM or above, vinblastin enhanced [$^3$H]PEP-Cu(II) uptake as shown by Fig. 6, whereas colchicine at 2 mM had no effect.

Since it is known that BLM is hydrolyzed to deamide BLM by an intracellular enzyme, BLM hydrolase[8,9], the formation of deamide PEP from intact PEP taken up into either AH66 or AH66F cells was examined by HPLC. Deamide PEP was detected from both AH66 and AH66F cells in-

Fig. 6. Effect of vinblastin on [$^3$H]PEP-Cu(II) uptake into AH66F.

The uptake was measured by incubating cells with 0.5 $\mu$g/ml of [$^3$H]PEP-Cu(II) for 5 minutes (○) or 4 hours (●).

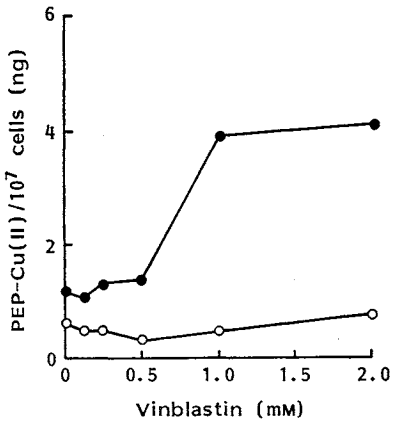
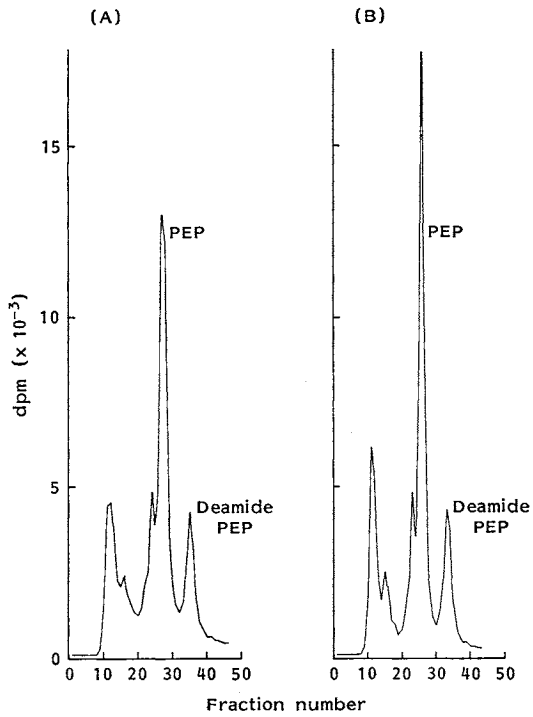



Fig. 7. Formation of deamide PEP from [$^3$H]PEP-Cu(II) taken up into AH66 (A) and AH66F (B) cells.




After tumor cells were incubated with 16 $\mu$g/ml of [$^3$H]PEP-Cu(II) for 4 hours, intact and deamide PEP were extracted from the cells and analyzed with high pressure liquid chromatography. Column: Nucleosil 50-5, eluent: $CH_3OH$ - $CH_3CN$ - 20% $CH_3COONH_4$ - $CH_3COOH$ (600 : 400 : 100 : 0.5). Fractions were collected at intervals of 30 seconds and counted with a liquid scintillation counter.

cubated with [$^3$H]PEP-Cu(II) for 4 hours (Fig. 7). The ratio of deamide PEP to intact PEP was about 1 : 3 in both tumor cells. Since deamide PEP was not found in the extracellular medium after 4 hours of incubation, it was concluded that deamide PEP was formed by the action of intracellular enzyme(s) on PEP taken up into tumor cells.

## Discussion

Although PEP and BLM have antitumor activity both *in vivo* and *in vitro*, the sensitivity to these drugs is very different among tumor cell lines[1,2]. AH66 and Ehrlich cells are very sensitive to PEP. AH66F, L1210 and P388 cells are insensitive[2]. Since the antitumor activity of PEP is thought to be due to the destruction of nuclear DNA, PEP has to permeate the cell membrane. It is possible, therefore, that the permeability to PEP is a factor determining the sensitivity of tumors. The time course of [$^3$H]PEP-Cu(II) uptake into tumor cells was biphasic with the first rapid uptake complete within 5 minutes (Fig. 1). The efflux after 5 minutes uptake was also rapid (Fig. 3). A similar rapid uptake was also observed in HeLa cells by ROY and HORWITZ[10], and L5178Y cells by UEHARA *et al.*[11], although they did not observed the second slow uptake. From these results and the linear dose-response of uptake, it is suggested that the rapid uptake represents the nonspecific association of [$^3$H]PEP-Cu(II) with the cell surface membrane. Deamide PEP was extracted from AH66 and AH66F cells incubated for 4 hours with [$^3$H]PEP-Cu(II). It has been demonstrated that BLM hydrolase which hydrolyses intact PEP into deamide PEP exists in the cytoplasm[8,9]. In addition, in the present study, the presence of deamide PEP could not be detected in the culture supernatant. Therefore, it is concluded that the second slow uptake represents the transport of [$^3$H]PEP-Cu(II) into the cells.

L1210 cells exhibited only the first rapid uptake. The second slow uptake into P388 cells reached saturation level in a short time. These results indicate that the insensitivity of L1210 and P388 cells

to PEP may be due to reduced uptake of PEP. The uptake of PEP into AH66 and AH66F cells increased linearly with incubation time. The uptake into AH66F cells was higher than into AH66 cells, although the sensitivity of AH66F cells to PEP was much lower than that of AH66 cells. In addition, the rate of inactivation of PEP into deamide PEP in the two cell lines was almost the same. These findings indicate that the difference between AH66 and AH66F cells in their sensitivity to PEP could not be ascribed to differences in uptake and inactivation.

The dose-response of [$^3$H]PEP-Cu(II) uptake after 4 hours incubation was linear up to the concentration of 200 $\mu$g/ml (Fig. 2), indicating that [$^3$H]PEP-Cu(II) transport is not mediated by a carrier system. However, this transport was dependent upon energy produced in mitochondria. Antitumor drugs such as doxorubicin, vincristine and etoposide are extruded in an energy-dependent process from multi-drug resistant cells[12,13]. However, the efflux of PEP was not influenced by metabolic inhibitors, and it was concluded that PEP influx was energy-suppressed. Hence, it is suggested that the permeability of the cell membranes to [$^3$H]PEP-Cu(II) is energy-dependent. This is supported by the observation that cell membrane modifiers such as dibucaine and chlorpromazine at concentrations of 1 mM or above enhanced [$^3$H]PEP-Cu(II) uptake. These agents are reported to interact with cell membrane and increase the fluidity of lipid membranes[7]. Vinblastin also enhanced [$^3$H]PEP-Cu(II) uptake, but since colchicine had no effect, this enhancement may not be due to an effect on microtubules.

Copper-free BLM is converted into deamide BLM by BLM hydrolase, but BLM-Cu(II) is not. Therefore, the detection of deamide PEP in AH66 and AH66F cells indicates that copper-free PEP was produced from PEP-Cu(II) within tumor cells. TAKAHASHI *et al.* reported that copper is reductively removed from BLM-Cu(II) by the cytosol fraction of AH66 cells[8]. Thus, the present result confirms that copper can be removed from BLM-Cu(II) in intact tumor cells.

In conclusion, the present results suggest that PEP-Cu(II) is taken up into tumor cells *via* a diffusion process which is controlled by an energy-dependent cell membrane barrier. Once inside the cell, the copper is removed and the drug partially metabolized to deamide PEP by BLM hydrolase.

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