ENHANCEMENT OF MITOMYCIN C UPTAKE BY ISOPROTERENOL IN RAT ASCITES HEPATOMA

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The amounts of mitomycin C (MMC) taken up into rat ascites hepatoma cells were determined by measuring the decrease in the absorbance of the incubation medium at 363 nm. The extracellular concentration of MMC decreased progressively in AH130 cell suspension and no peaks other than MMC were detected by analytical high-performance liquid chromatography (HPLC). Isoproterenol (IPN) enhanced the uptake of MMC into AH44 and AH130 cells but not AH13 cells in which the uptake increased by N\(^\circ\),O\(^{\circ}\)-dibutyryl cyclic adenosine 3':5'-monophosphate (dibutyryl cyclic AMP). The increase of uptake of MMC by IPN was inhibited by propranolol and the uptake of MMC increased in a dose-dependent manner by theophylline in AH130 cells. The maximum combined cytotoxicity was observed when AH130 cells were treated with IPN at 10\(^{-7}\) M for 30 min before the exposure to MMC and, in this pretreatment condition, the uptake of MMC was enhanced in parallel with the increase of cyclic adenosine 3':5'-monophosphate (cyclic AMP) level in the cells. On the other hand, MMC, which had no stimulating effect on the intracellular cyclic AMP level, nevertheless maintained a high level of intracellular cyclic AMP elevated by IPN for a longer period than in the treatment with IPN alone and prolonged the period of acceleration of the uptake of MMC. In the in vivo combined treatment with IPN and MMC, the life span of AH44-bearing rats was prolonged and 2 out of 6 rats were cured, while the mean survival time of the rats treated with MMC alone was 11.3 d. These results indicated that the intracellular cyclic AMP, which is maintained at a high concentration for a long period by the combined action of IPN and MMC, enhanced the uptake and the antitumor effect of MMC.

Keywords — rat ascites hepatoma; isoproterenol; cyclic AMP; mitomycin C; uptake; combination effect

INTRODUCTION

Potentiation of the effect of antitumor agents is an important problem in cancer chemotherapy. Many investigators have shown that inactive antitumor agents enhance the effect of antitumor agents and proposed the mechanism of their combination effects.\(^1\) Our recent studies have indicated that isoproterenol (IPN), a \(\beta\)-adrenergic stimulant, synergistically potentiated the cytotoxic effect of mitomycin C (MMC) on HeLa cells and some rat ascites hepatoma cells, by this was not so for AH13 cells whose adenylate cyclase was hardly activated by IPN.\(^2\) Therefore, it was suggested that the endogenous cyclic adenosine 3':5'-monophosphate (cyclic AMP) elevated via \(\beta\)-adrenoceptors by IPN potentiated the effect of MMC.

This study describes the relationship among the antitumor effect, the cyclic AMP level and the uptake of MMC in rat ascites hepatoma treated with IPN and MMC.

MATERIALS AND METHODS

Chemicals — The materials used were mitomycin C (MMC, Sankyo Co., Tokyo), \(d,l\)-isoproterenol hydrochloride (IPN, Nakarai Chemicals, Ltd., Kyoto), theophylline (Wako Pure Chemical Ind., Osaka), N\(^\circ\),O\(^{\circ}\)-dibutyryl cyclic adenosine 3':5'-monophosphate (dibutyryl cyclic AMP) and \(d,l\)-propranolol hydrochloride (Sigma Chemicals Co., St. Louis, U.S.A.).

Tumor Cells — Three lines of rat ascites hepatoma cells were used in this study. Cells were maintained by the serial intraperitoneal passage at weekly intervals in female Donryu rats (weight 100−150 g, Shizuoka Laboratory Animal Center). The cells were withdrawn from the abdominal cavity of each rat 5 to 9 d after their implantation. The cells were used in the experiments.

Cell Culture — AH130 cells were suspended in Eagle’s minimum essential medium (Eagle’s MEM) to a concentration of about 4 × 10\(^5\) cells/
ml and treated with MMC (0.03 μg/ml) for 30 min after addition of IPN at 37 °C. The treated cells were washed with Hanks’ solution, suspended in Eagle’s MEM containing 10% fetal calf serum and cultured in 35-mm plastic petri dishes (Falcon 3001) in a CO₂ incubator for 2 d at 37 °C. The viable cells were assayed by the staining method using 0.2% Trypan Blue.

**In Vivo Combined Experiment** — Female Donryu rats (6 weeks old) were intraperitoneally inoculated with 1 × 10⁶ AH44 cells. At 3 d after the cell inoculation, IPN was intraperitoneally injected into the rats and after 15 min MMC was also intraperitoneally administered.

**Determination of Cyclic AMP Content** — The washed cells were suspended in phosphate-buffered saline (PBS) to a concentration of about 5 × 10⁶ cells/ml and treated with the test materials at 37 °C. After the treatment, they were chilled for 5 min and centrifuged. The intracellular cyclic AMP was extracted by homogenizing the cells in 6% trichloroacetic acid and the extract was assayed with a cyclic AMP assay kit (Yamasa Shoyu K.K., Choshi) following the procedure developed by Honma et al. ³³

**Determination of MMC Amount** — Cells were treated with the test materials in PBS at 37 °C and then exposed to MMC. After the treatment, they were chilled, centrifuged and after removal of the supernatant fluid, the cells were sonicated in methanol. The sonicated cells were kept overnight at 4 °C and centrifuged to obtain a methanol extract. The amounts of MMC in the supernatant fluid or in the methanol extract were measured by the methods mentioned below.

i) Direct Spectrophotometry: MMC amounts were determined by measuring the absorbance of the quinone form of the agent at 360 nm (Hitachi 100—20 spectrophotometer) using a calibration curve.

ii) Analytical High-Performance Liquid Chromatography (HPLC) ⁴¹: a Yanaco L-5000 liquid chromatograph equipped with a Yanaco M-315 spectrometer was used with a reversed phase Yanpak ODS-A stainless column (25 cm × 4.6 mm i.d.). A mobile phase consisting of 0.01 M phosphate buffer (pH 6.0)–methanol (70:30, v/v) was filtered through a 0.45 μm filter (Toyo Roshi Co., Ltd.) and degassed ultrasonically before use. The flow rate was 1.0 ml/min. The wave length of the detector was 365 and 313 nm. Samples were filtered and injected in volume range of 10 to 50 μl. The retention time of MMC was 14.4 min at room temperature. The amounts were calculated from peak height using a calibration curve. The calibration curves of MMC obtained from the two methods were linear up to 40 μg/ml. A direct spectrophotometry was used to determine the MMC amounts, unless otherwise indicated.

**RESULTS**

**Uptake of MMC**

Figure 1 shows the HPLC elution profiles of incubation fluids and the cell extracts and Fig. 2 shows the time course of the disappearance of extracellular MMC in cultures of AH130 cells measured by a direct spectrophotometry and HPLC. When AH130 cells were incubated with MMC (10 μg/ml), the concentration of extracellular MMC decreased progressively for 120 min. During the experimental period no peaks other than MMC were observed in the incubation medium by HPLC analysis (Fig. 1a—c).

![FIG 1. High-Performance Liquid Chromatograms of MMC in the Supernatant Fluids of Cultures of AH130 Cells and the Cell Extract](image-url)

**HPLC analyses** of the supernatant fluids of cell suspensions (10⁷ cells/ml) treated with MMC (10 μg/ml) (A) and the methanol extracts of cells (1.6 × 10⁸ cells/2 ml) treated with MMC (40 μg/ml) at 37°C for the indicated time (B).
Enhancement of MMC Uptake by Isoproterenol

When MMC was incubated in the absence of cells, no changes in the concentration and in the elution profile of HPLC were observed, even after 60 min (Fig. 1d). On the other hand, while only a small amount of unchanged MMC was detected in the cells, about six peaks which appeared to be metabolites of MMC rapidly increased with the time of incubation (Fig. 1e–g). It is known that MMC, which itself is inactive, is activated enzymatically by microsomes and nuclei to form alkylating species. Therefore, the amount of MMC taken up into the cells in the following experiments was calculated from the amount of decrease of MMC in the incubation medium.

**Effect of IPN and Dibutyryl Cyclic AMP on the Uptake of MMC**

When MMC (10 μg/ml) was incubated with IPN (10⁻⁷ M) or dibutyryl cyclic AMP (10⁻⁵ M) without tumor cells at 37 °C, no reaction products appeared and the concentration of MMC in the incubation medium did not change for 60 min (data not shown). As shown in Fig. 3, when MMC was added to each tumor cell suspension, MMC was progressively taken up into AH130, AH44 and AH13 cells during the 60 min period. The amount of MMC taken up by AH13 cells was least. IPN (10⁻⁷ M) accelerated the initial rate of MMC uptake by AH44 and AH130 cells but not by AH13 cells. Dibutyryl cyclic AMP (10⁻⁵ M) also increased the uptake of MMC in AH130 AH44 cells and even in AH13 cells. Furthermore, theophylline increased the uptake of MMC in a dose-dependent manner in AH130 cells (Fig. 4).

**Influence of Propranolol on the Effect of IPN in AH130 Cells**

Propranolol added simultaneously with IPN (10⁻⁷ M) inhibited the increase of MMC uptake by IPN in a dose-dependent manner and 10⁻⁵ M of propranolol almost negated the effect of IPN (Fig. 5).

**Comparison of the Effect of IPN on the Cytotoxicity**

![Figure 2: Time Course of the Disappearance of MMC from AH130 Cell Incubation Medium](image)

![Figure 3: Effect of IPN and Dibutyryl Cyclic AMP on the Uptake of MMC in Rat Ascites Hapatoma Cells](image)
FIG. 4. Effect of Theophylline on the Uptake of MMC in AH130 Cells
Cells (10⁷ cells/ml) were incubated with varying concentrations of theophylline for 15 min and then exposed to MMC (10 μg/ml) for 30 min.

FIG. 5. Influence of Propranolol on the Effect of IPN on the Uptake of MMC in AH130 Cells
Cells (10⁷ cells/ml) were incubated with IPN (10⁻⁷ M) and varying concentrations of propranolol for 15 min and then exposed to MMC (10 μg/ml) for 30 min. ●, with IPN; ○, without IPN.

FIG. 6. Effect of IPN on the Cytotoxicity, the Uptake of MMC and Intracellular Cyclic AMP Level in AH130 Cells
The cytotoxicity was assayed 2 d after the treatment with IPN and MMC (0.03 μg/ml). The intracellular cyclic AMP contents were determined in the cells treated with IPN. The amount of MMC taken up into the cells was measured by the incubation with MMC (10 μg/ml) for 30 min after addition of IPN. Left panel: MMC was added at 15 min after addition of varying concentrations of IPN. Right panel: MMC was added at the indicated time after addition of IPN (10⁻⁷ M).
and the Uptake of MMC and the Intracellular Cyclic AMP in AH130 Cells

Figure 6 shows the effects of IPN in AH130 cells. The pretreatment with IPN for 15 min synergistically potentiated the cytotoxicity of MMC. Using the same condition of pretreatment with IPN, the intracellular cyclic AMP level increased about 50% over the non-treated level and the uptake of MMC was also increased. The maximum effect was observed at 30 min after the addition of 10^{-7} M of IPN for each assay. Thus, it was clear that the potentiation of cytotoxicity of MMC by IPN was closely related to the increase of intracellular cyclic AMP level and the enhancement of MMC uptake into the cells. On the other hand, the cyclic AMP level increased by IPN in AH130 cells was maintained for a long period by MMC, and even at 120 min after the start of IPN treatment, cyclic AMP level in the cells was maintained 1.5-fold of the basal level in the non-treated cells. In this period, the cells possessed the capacity to take up more MMC than non-treated cells (Fig. 7).

Combination Therapy with IPN and MMC in AH44-Bearing Rats

It is thought that combination effect may be more easily observed in cells resistant to antitumor agents than in sensitive cells. AH44 used in this experiment is a cell line not sensitive to alkylating agents. The life span of AH44-bearing rats was markedly prolonged by only a single combined treatment with IPN (0.1 mg/kg) and MMC (0.2 mg/kg) and 2 out of 6 rats were cured, whereas the rats treated with MMC alone died within 19 d after tumor cell inoculation and the mean survival time was 11.3 d (Fig. 8). The experiments using larger dosages of these agents were not successful because that the adverse effects of IPN and the antitumor effect of MMC were much strong.

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**FIG. 7. Combined Effect of IPN and MMC on the Intracellular Cyclic AMP Level and the Uptake of MMC in AH130 Cells**

In the upper panel, cells were treated for 30 min without (○) or with MMC (0.1 μg/ml) (●) at 15 min after addition of 10^{-7} M IPN. The treated cells were washed, resuspended to the original volume with PBS and were further incubated. The intracellular cyclic AMP contents were measured in the cells incubated for the indicated time. In the lower panel, cells were treated without (○) or with MMC (1 μg/ml) (●) at 15 min after addition of 10^{-7} M IPN. MMC (10 μg/ml) was further added to the cell suspension at the indicated time and MMC taken up into the cells for 30 min was measured.

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**FIG. 8. Combined Antitumor Effect of IPN and MMC on AH44-Bearing Rats**

Six rats in a group were inoculated intraperitoneally with 1 × 10^6 AH130 cells and 3 d after the cell inoculation, IPN (0.1 mg/kg) was intraperitoneally injected at 15 min before a single administration of MMC (0.2 mg/kg) (1).

-- , control; ---- , IPN; --- , MMC; -------- , IPN and MMC.
DISCUSSION

We have determined, with an analytical HPLC method, that the concentration of extracellular MMC decreased when this agent was incubated with tumor cells (Fig. 2) and that it was stable in PBS solution. In the extract of AH130 cells, several metabolites of MMC increased in the course of incubation time and only a trace of the unchanged form was present (Fig. 1). Namely, this indicated that the disappearance of MMC from the cell suspension was due to its uptake by the cells and then it was rapidly metabolized. MMC was progressively taken up into AH130, AH44 and AH13 cells. The amount of MMC taken up during 60 min was greatest by AH130 cells, next by AH44 cells and least by AH13 cells. The sensitivities of these tumors to MMC were in the order of AH130 > AH13 > AH44. Therefore, there appeared to be no relationship between the amounts of MMC taken up into these rat ascites hepatoma cells and sensitivities to the agent. Their sensitivities may be due to the difference in their repair capacities of deoxyribonucleic acid impaired by the agent and in the activities of enzymes activating or degrading MMC.

Our previous papers have suggested that the combined cytotoxic effect of β-adrenergic stimulants with some alkylating agents was closely related to the increase of intracellular cyclic AMP. In the present paper, we investigated the effect of IPN or dibutyryl cyclic AMP on the cellular uptake of MMC using rat ascites hepatoma cells. There is evidence that catecholamines or cyclic AMP regulates the membrane transport of glucose, amino acids, nucleotides and ions. Galivan reported that glucagon increased the rate and the extent of accumulation of methotrexate which was transported through the cell membrane in an energy-dependent manner, and the effect of glucagon appeared to be acting, in part, through the adenylate cyclase system in primary cultured liver cells. In contrast, the rate of the transport of methotrexate in L210 cells was diminished by increasing levels of cyclic AMP. On the other hand, White et al. reported that the effect of cyclic AMP on the transport of methotrexate might not be a general phenomenon. The influence of cyclic AMP on the membrane transport of MMC has not been described. In this study, IPN augmented the uptake of MMC into AH44 and AH130 cells but not into AH13 cells in which cyclic AMP was not increased and adenylate cyclase was not stimulated by IPN. However, dibutyryl cyclic AMP enhanced the uptake of MMC in all cell lines (Fig. 3). The augmentation of MMC uptake by IPN in AH130 cells was negated by propranolol, a β-adrenergic antagonist (Fig. 5), and the uptake was augmented by theophylline (Fig. 4). Furthermore, the enhancement of cytotoxicity of MMC by IPN was in parallel with the increase of intracellular cyclic AMP levels and MMC uptake by IPN (Figs. 6, 7). This study also showed that MMC prolonged the term of the high cyclic AMP level elevated by IPN and of augmentation of its uptake (Fig. 7). These results indicate that increased cyclic AMP in rat ascites hepatoma cells augments the transport of MMC and potentiates the cytotoxicity of the antitumor agent. Additionally, this study indicated that the combination effect of IPN and MMC was also effective in vivo (Fig. 8). This suggested a possibility of a practical application for antitumor combined chemotherapy.

Ishikawa determined the amount of MMC taken up into Ehrlich ascites tumor cells by measuring the absorbance of the cell extract at 360 nm and suggested that MMC was taken up by an energy-independent system. However, because in this study MMC was hardly detectable as an unchanged form in AH130 cells, this evidence may not be available to elucidate the transport of MMC in rat ascites hepatoma cells. It has been reported that MMC was activated and metabolized mainly by microsomal enzymes. Cyclic AMP activates the cellular metabolic abilities by the phosphorylation of proteins of various kinds. If cyclic AMP influences the metabolism and the transport of MMC, the mechanism of the increase of MMC influx by IPN may be clarified. Further studies on the action of cyclic AMP on the transport system and metabolism of MMC are in progress.

REFERENCES

Enhancement of MMC Uptake by Isoproterenol


