Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 31-37

EFFECTS OF THE COMBINATION OF ACIVICIN AND cis-DIAMMINE-DICHLOROPLATINUM(II) ON THYMIDYLATE SYNTHESIS OF A549 LUNG CANCER CELLS

Hitoyasu Futami, Taiichi Shiotani, Yasufumi Yamaji, Noriko Yamauchi and Shozo Irino*

First Department of Internal Medicine
Kagawa Medical School, Ikenobe, Miki, Kagawa 761-07, Japan

Received April 12, 1989

Summary. Compared to either compound alone, the combination of acivicin and cis-diamminedichloroplatinum(II) markedly enhanced the inhibition of the activities of thymidylate synthase and thymidine kinase, the enzymes involved in the final steps of the de novo and salvage pathways in pyrimidine metabolism in A549 lung cancer cells. The enhancement of enzymic inhibition paralleled that of cell growth inhibition. These results indicate that the combination of these drugs can inhibit the capacities of the pyrimidine pathways, resulting in an efficient reduction of DNA synthesis.

Acivicin, an L-glutamine antagonist, has been shown to inhibit several glutamine-dependent amidotransferases including GMP synthase, carbamoyl phosphate synthase II and CTP synthase (1,2) and thereby blocks de novo purine and pyrimidine synthetic pathways. However, little information is available about the effect of acivicin on thymidine metabolism, which would be most relevant for understanding the role of DNA synthesis in cancer cells. Thymidylate, an important precursor of DNA synthesis, may be produced not only by a de novo pathway through the rate-limiting enzyme dTMP synthase (EC 2.1.1.45), but also by salvage through thymidine kinase (EC 2.7.1.21), which catalyzes the final reaction in the salvage pathway (3). CDDP has been demonstrated to have powerful antineoplastic activity

*To whom correspondence should be addressed.

Abbreviations used: CDDP, cis-diamminedichloroplatinum(II); CTP, cytidine triphosphate; dTMP, thymidylate; dTTP, thymidine triphosphate; GMP, guanylate; IC_{50}, 50% inhibitory concentration.

0006-291X/89 $1.50

against various kinds of cancer cells (4). Although the antineoplastic mechanism of CDDP has been explained by the formation of DNA cross-links (5), CDDP appears to have certain effects on pyrimidine metabolism (6-8). Furthermore, we have demonstrated recently that CDDP inhibited the capacities of the salvage pathway of pyrimidine metabolism in A549 lung cancer cells (9). Therefore, it is of great interest to examine whether the combination of acivicin and CDDP, as inhibitors of de novo and salvage pyrimidine biosynthesis, respectively, would enhance the inhibition of DNA synthesis.

In this study, we investigated the inhibitory effects of acivicin alone and in combination with CDDP on the growth of A549 lung cancer cells and the activities of dTMP synthase and thymidine kinase.

MATERIALS AND METHODS

Chemicals. Acivicin was kindly provided by Dr. G. Weber (Laboratory for Experimental Oncology, Indiana University, Ind. USA). CDDP was obtained from Nihon Kayaku Co., Ltd.(Tokyo, Japan). Acivicin and CDDP were dissolved in distilled water and diluted with fresh medium to the appropriate concentrations. (5-^{3}H)Deoxyuridylate (8.9 Ci/mmol) and ACS II were purchased from Amersham Corp. (Arlington Heights, Il.). (Methyl-^{3}H)thymidine (20.0 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). DMEM and F12 culture medium were from GIBCO (Grand Island, N.Y.). All other reagents were also of the highest available analytical grade.

Cells and cell culture. A549 human lung cancer cells (10), obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were maintained in monolayers in the logarithmic phase of growth in DMEM/F-12 medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37 °C. The doubling time was 36 ± 2 h, respectively. The cultured cells were removed from the substrate by the treatment with 0.05% trypsin for 5 min at 37 °C, harvested by centrifugation and washed twice with phosphate-buffered saline.

Growth inhibition. Exponentially growing cells were seeded at a density of 1×10^5 cells/ml in 10 ml of medium in a plastic dish (100 x 20 mm; Becton Dickinson, Lincoln Park, N.J.) with a plating efficiency ranging from 35 to 70%. Twenty-four h after seeding, various concentrations of acivicin and/or CDDP were added to the medium. After incubating for the indicated times, the medium was discarded, and the cell number of the trypsinized cells were counted by trypan blue dye exclusion. Duplicate cultures were carried out for each experiment.

Enzyme assays. Preparation of cytoplasmic extracts from cultured cells and the assay methods for dTMP synthase and thymidine kinase activities and protein contents were described elsewhere (3,11). Enzyme activity is expressed as nmol of product formed/h/mg protein.

RESULTS

Growth inhibition by acivicin. A549 cells were continuously exposed to various concentrations of acivicin (from 10^{-7} to 10^{-4} M) for 96 h (Fig. 1). Total cell numbers of untreated cells cultured for 48 and 96 h were 5.73 ± 1.99 x 10^6 and 11.72 ± 3.56 x 10^6 cells, respectively. No cytotoxic effect was elicited for cells incubated for 96 h with 10^{-7} M acivicin. However, cell growth was inhibited at concentrations above 10^{-6} M in a time-dependent manner. Treatment with 10^{-6} or 10^{-5} M acivicin for 48 h retarded cell growth by 32 or 68% of the control, and treatment for 96 h inhibited it by 51 or 79%, respectively. No significant difference in the extent of inhibition was observed between 10^{-5} and 10^{-4} M acivicin. The IC_{50} value with exposure for 48 h was 4.5 ± 0.5 x 10^{-6} M.

Enhancement of acivicin-growth inhibition by CDDP. The effect of CDDP on acivicin-induced growth inhibition is shown in Fig. 2. Both drugs were added simultaneously to the medium, and cells were cultured for 48 h. CDDP alone inhibited cell growth in a dose-dependent manner; 8, 54 and 74% inhibition were observed with exposure to 10^{-6}, 2 x 10^{-6} (data not shown) and

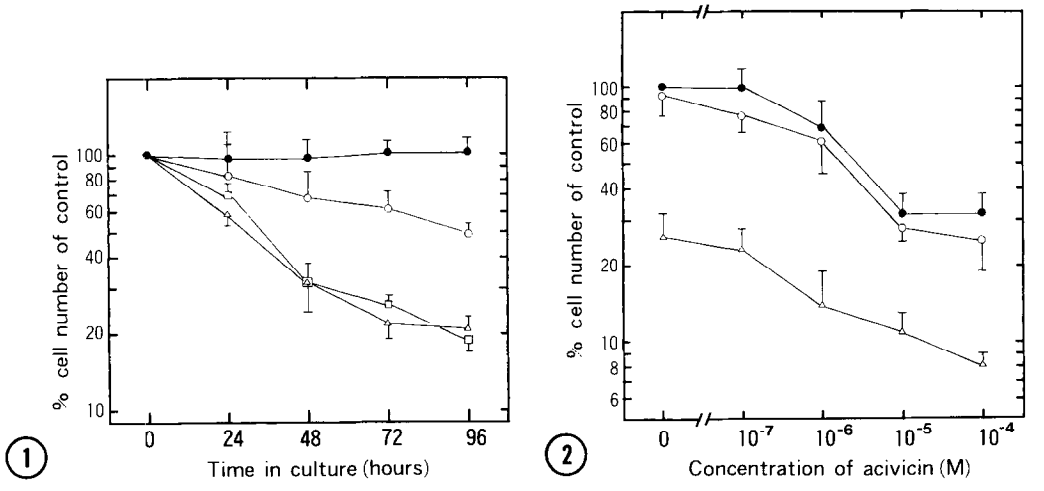

Fig. 1: Time-course of inhibition of A549 cell growth by different concentrations of acivicin. A549 cells were cultured in the absence or presence of 10^{-7} M (●), 10^{-6} M (○), 10^{-5} M (△) or 10^{-4} M (□) acivicin for the indicated times. Results are expressed as the percentage of control values for 4 separate determinations.

Fig. 2: Effect of acivicin and CDDP on the growth of A549 cells. A549 cells were exposed to various concentrations of acivicin alone (●) and in combination with CDDP at 10^{-6} M (○) and 10^{-5} M (△) for 48 h. Results were evaluated as in Fig. 1.

10^{-5} M CDDP, respectively. The IC_{50} value of CDDP was 3.0 ± 0.6 x 10^{-6} M. Addition of 10^{-5} M CDDP in combination with acivicin resulted in a more than additive inhibitory effect on the cell growth, while 10^{-6} M CDDP produced little additional effect. Survival rates of the cells treated with 10^{-6} , 10^{-5} or 10^{-4} M acivicin were reduced from 68 to 14%, 32 to 11% or 32 to 8% by the addition of 10^{-5} M CDDP.

Effect of the combination of acivicin and CDDP on enzyme activities. Effects of acivicin alone and in combination with CDDP on the activities of dTMP synthase and thymidine kinase were examined (Fig. 3). Acivicin alone inhibited both enzyme activities in a dose-dependent manner. An exposure to 10^{-4} M acivicin for 48 h reduced the activities of dTMP synthase and thymidine kinase to 31 and 51% of the activities in the untreated cells, respectively. CDDP also inhibited both enzyme activities in a similar manner. Exposure to 10^{-5} M CDDP retarded thymidylate synthase and thymidine kinase activities to 61 and 60% of the controls. The absolute specific activities of dTMP synthase and thymidine kinase of untreated cells at 24 h after seeding were 8.41 ± 1.78 and 0.36 ± 0.09 nmol/h/mg protein, respectively. The combination of acivicin and CDDP caused a marked inhibition of both enzyme activities compared to that produced by acivicin or CDDP alone. The concentrations of acivicin and

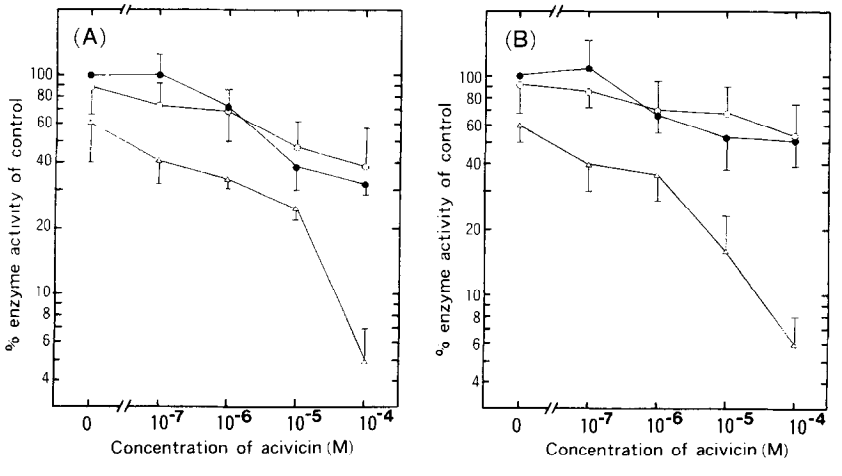

Fig. 3: Effect of acivicin and CDDP on enzyme activities. A549 cells were exposed to various concentrations of acivicin alone (●) and in combination with CDDP at 10^{-6} M (○) or 10^{-5} M (△) for 48 h. The activities of dTMP synthase (A) and thymidine kinase (B) were determined in the cytoplasmic extracts of A549 cells. Activities were calculated in nmol/h/mg protein, and results are shown as a percentage of the control activities.

CDDP resulting in the greatest enhancement of the inhibition of enzyme activities were 10^{-4} and 10^{-5} M, respectively; dTMP synthase activity decreased from 31 to 5% of the control (Fig. 3A), and the activity of thymidine kinase declined more profoundly from 51 to 6% (Fig. 3B), indicating that thymidine kinase activity was preferentially inhibited. Significant enhancement of the inhibition of both enzyme activities was not observed with the addition of 10^{-6} M CDDP. The extents of inhibitory activities calculated per cell number basis were essentially the same as those per mg protein basis (data not shown). The enhancement of inhibitory activity towards both enzyme activities by the combination of acivicin and CDDP paralleled their inhibitory activity towards cell growth (Fig. 2).

DISCUSSION

The present study clearly demonstrated that acivicin inhibited the activities of dTMP synthase, the rate-limiting enzyme in the last step of the de novo pathway, although this agent has been shown to inhibit L-glutamine-dependent enzymes in pyrimidine de novo synthesis (1,2). The inhibition of dTMP synthase by acivicin might be accounted for by the depletion in the levels of deoxyuridylate, the substrate for the dTMP synthase reaction (1,2). It is also clear that CDDP could inhibit both dTMP synthase and thymidine kinase activities; this may be partly explained by the fact that CDDP can inhibit amino acid transport in cancer cells, thereby affecting the intracellular contents of the co-factors of these enzymes (8). The inhibitory action of acivicin or CDDP might not be due to a direct effect on the enzyme protein, because enzyme activities in cell-free extracts were not inhibited by these drugs (data not shown). Since dTMP, an important precursor of DNA synthesis, may be produced not only by de novo but also through salvage pathways, the marked increases in the enzymic capacities for the salvage pathway in tumors (2,3,12,13) account, at least in part, for the lack of chemotherapeutic success of inhibitors of enzymes of de novo synthesis. These observations emphasize the importance of the salvage pathway and suggest that the combination of antimetabolites of the de novo pathway and inhibitors or blockers of salvage enzymes should improve chemotherapeutic effectiveness (2,3). Zhen et al. demonstrated that the combination of acivicin and dipyridamole, an inhibitor

of the salvage pathway, yielded synergistic anticancer cytotoxicities (14). Therefore, acivicin and CDDP, in combination, would be ideal on the biochemical basis of pyrimidine metabolism. Our data indicate that the combination of acivicin and CDDP markedly enhanced the inhibition of dTMP synthase and thymidine kinase activities, which was accompanied by a more than additive cytotoxic effect on A549 cell growth. Inhibition of thymidine kinase activity was preferentially enhanced at concentrations over 10^{-5} M acivicin and CDDP compared to that of dTMP synthase, resulting in a possible decline of dTTP production through the salvage pathway and consequently, causes a reduction in DNA biosynthesis. A lower enhancement of the capacities of the de novo pathway (dTMP synthase activity) also might affect DNA synthesis to a significant extent.

The combination of CDDP and an antimetabolite such as 5-fluorouracil (8) or cytosine arabinoside (6,7) has been shown to have synergistic activities against cancer cells. Although the mechanisms of synergism have not been elucidated in detail, the present observations suggest that those synergisms may be attributable, at least partly, to an enhancement by CDDP of the inhibition of thymidine kinase in the salvage pathway.

In conclusion, our studies indicate that CDDP can potentiate the cytotoxic action of acivicin on A549 cells, producing an enhancement of the inhibition of pyrimidine synthetic capacities. This combination should have potential impact on the treatment of neoplastic diseases.

REFERENCES

1. Earhart, R. H. (1987) In Concepts, Clinical Developments, and Therapeutic Advances in Cancer Chemotherapy (F. M. Muggia, Ed.), pp. 161-181. Martinus Nijhoff Publishers, Boston, MA.
2. Weber, G. (1983) Cancer Res. 43, 3466-3492.
3. Shiotani, T., Hashimoto, Y., Tanaka, T., and Irino, S. (1989) Cancer Res. 49,1090-1094.
4. Wolpert-DeFilippes, M. K. (1979) Cancer Treat. Rep. 63, 1453-1458.
5. Zwelling, L. A., and Kohn, K. W. (1979) Cancer Treat. Rep. 63, 1439-1444.
6. Bergerat, J-P., Drewinko, B., Corry, P., Barlogie, B., and Ho, H. (1981) Cancer Res. 41, 25-30.
7. Kern, D. H., Morgan, C. R., and Hildebrand-Zanki, S. U. (1988) Cancer Res. 48, 117-121.
8. Scanlon, K. J., Newman, E. M., Lu, Y., and Priest, D. G. (1986) Proc. Natl. Acad. Sci. USA. 83, 8923-8925.

9. Yamaji, Y., Yamauchi, N., Futami, H., Shiotani, T., and Irino, S. (1987) Proc. Am. Assoc. Cancer Res. 28, 312.
10. Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H., and Parks, W. P. (1973) JNCI 51, 1417-1421.
11. Hashimoto, Y., Shiotani, T., and Weber, G. (1987) Anal. Biochem. 167, 340-346.
12. Weber, G., Shiotani, T., Kizaki, H., Tzeng, D., Williams, J. C., and Gladstone, N. (1978) Adv. Enzyme Regul. 16, 3-19.
13. Hashimoto, Y., Shiotani, T., Eble, J. N., Glover J. L., and Weber, G. (1988) Cancer Biochem. Biophys. 10, 1-10.
14. Zhen, Y., Lui, M. S., and Weber, G. (1983) Cancer Res. 43, 1616-1619.