Jpn. J. Cancer Res. (Gann), 79, 1230–1237; November, 1988

Impairment of Methotrexate (MTX)-Polyglutamate Formation of MTX-resistant K562 Cell Lines

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We examined the mechanism of methotrexate (MTX) resistance in five K562 cell subclones resistant to MTX. Based on a clonogenic assay, the IC_{50}s of these MTX-resistant clones were 10 to 40μM MTX, indicating 2,000 to 5,000-fold resistance as compared to that of the parent cell line. The doubling times of these MTX-resistant K562 cell lines are longer (27–60 hr) than that of the parent K562 cell line (24 hr). One-hour MTX accumulation in the resistant cells was 70–80% of that in parent cells. To investigate the formation of MTX-polyglutamates (MTX-PGs), resistant cells were incubated with ^{3}H-MTX (1 or 10μM) for 24 hr in the presence of thymidine and deoxyinosine to prevent cytotoxicity. MTX (-Glu_1) and the polyglutamate metabolites (MTX-Glu_2, -Glu_3, -Glu_4 and -Glu_5) were analyzed by a high-pressure liquid chromatography (HPLC) technique. After a 24-hr incubation with 10μM MTX, the total concentration of intracellular MTX reached 39 to 89 nmol/g protein, only 20 to 40% of the MTX level of the parent K562 cells. The HPLC analysis revealed that less than 2% of intracellular MTX was in the form of high-molecular MTX-PGs (MTX-Glu_3, -Glu_4 and -Glu_5) in the five MTX-resistant K562 cell lines, while in the parent cells MTX-Glu_{3-5} comprised 46% of the total intracellular MTX. These data indicate the possibility that impairment of MTX-PG formation, with transport alteration, may be a special mechanism for the high level of resistance to this agent in human leukemic cells.

Key words: Methotrexate — Polyglutamation — Drug resistance — K562 cells

Although methotrexate (MTX) is useful in the treatment of a variety of human malignancies, the development of MTX resistance in the clinical setting has become a common problem. Several mechanisms of resistance of malignant cells to this drug have been established in experimental systems[1-3]: (a) decreased membrane transport,[4-6] (b) alterations in the binding affinity of dihydrofolate reductase (DHFR) for MTX,[7-9] (c) increased levels of DHFR due to gene amplification,[6, 10, 11] and (d) decreased activity of thymidylate synthase (TS).[10, 12-14] Recent biochemical studies revealed that MTX is metabolized to poly-γ-glutamyl derivatives containing two to five residues, MTX-polyglutamates (MTX-PGs), which are preferentially retained and which block the target enzyme, DHFR as well as other important folate-dependent enzymes such as 5-aminoimidazole carboxamide ribotide transformylase (AICAR T'ase) and TS.[1, 15-17] Polyglutamation of MTX takes place in both normal and malignant cells, including human erythrocytes,[18] liver,[19] bone marrow myeloid precursors[20] and fibroblasts,[21] as well as murine tissues,[22] leukemia cells,[23] hepatoma cells,[24] human breast cancer cells,[25] human small cell carcinoma cells,[26] and various other human and murine neoplasms.[27, 28] From these studies, decreased metabolism of MTX to form MTX-PGs has been suggested as another mechanism of MTX resistance.[28-32] In this study we developed five MTX-resistant subclones of the K562 cell line, which itself was established from a patient with erythroblastoid crisis of human chronic myelogenous leukemia, and the MTX-polyglutamation profiles of these resistant clones were investigated. Impairment of MTX-PG formation was found to be a possible major mechanism of MTX resistance.

The abbreviations used are: MTX, methotrexate; MTX-PGs, methotrexate-polyglutamates; AICAR T'ase, 5-aminoimidazole carboxamide ribotide transformylase; DHFR, dihydrofolate reductase; TS, thymidylate synthase.

Materials and Methods

Establishment of MTX-resistant K562 Cell Line The K562 cell line was obtained from the American Type Culture Collection (Rockville, MD) and was grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, M. A. Bioproducts, Walkersville, MD), 2m*M* glutamine (Nissui, Tokyo), 100 units/ml penicillin and 5 μg/ml gentamicin at 37° in a humidified atmosphere of 5% CO_2. MTX was obtained from the Drug Synthesis and Chemistry Branch, NCI (Bethesda, MD). The MTX-resistant subclones (K562/MTX) of K562 cells were derived from the parent line by exposure of cells to gradually increasing concentrations of MTX *in vitro*. Initially, K562 cells (2×10^4 cells/ml) were treated with $3\times10^{-9}M$ MTX. The concentration of MTX was gradually increased every 2–3 weeks until the concentration finally reached $5\times10^{-6}M$ MTX after approximately 6 months. MTX-resistant cultures were then cloned in growth medium containing $10^{-6}M$ MTX by a limiting dilution technique. Cells were diluted to one cell per 0.4 ml of the growth medium and 0.2 ml was distributed into each of 96 wells in a Multiwell plate (Falcon, Becton-Dickinson, Lincoln Part, NJ). After incubation for 1 week, wells containing a single colony were marked. Those cells which contained 2 or more clones were discarded. After sufficient growth, five cloned cell lines (K562/MTX-1, -2, -3, -4 and -5) were finally established. They have so far been maintained for more than 12 months in complete medium containing $1–5\times10^{-6}$ *M* MTX.

Cytotoxicity Studies The effects of drug exposure were determined using a clonogenic assay. The parent K562 and resistant K562/MTX clones (10^4 cells) were plated in 1 ml of 0.9% methylcellulose (Fisher, Fair Lawn, NJ) in complete medium containing various concentrations (10^{-9} to $10^{-4}M$) of MTX. After 7–10 days of incubation at 37° under 5% CO_2, colonies were counted using an inverted microscope.

Determination of Intracellular MTX and MTX-PGs To examine intracellular contents of MTX and MTX-PGs, the parent K562 and MTX-resistant K562/MTX cells were first incubated in complete medium without MTX for 72 hr, and then they were incubated at the concentration of 10^6 cells/ml in RPMI 1640 with 10% dialyzed FBS and either 1 or 10μM ^{3}H-MTX (Amersham, Arlington Heights, IL). This incubation medium was further supplemented with 10μM thymidine (Wako, Tokyo) and 10μM deoxyinosine (Wako) to protect the cells from the direct cytotoxic action of MTX. At the end of an incubation period with MTX, cells were harvested to determine total intracellular drug and metabolite levels. Some of the cells were washed twice with PBS, resuspended in drug-free complete media and incubated for an additional 24 hr to determine drug efflux patterns.

The intracellular contents of MTX and MTX-PGs were determined by the method of Jolivet *et*

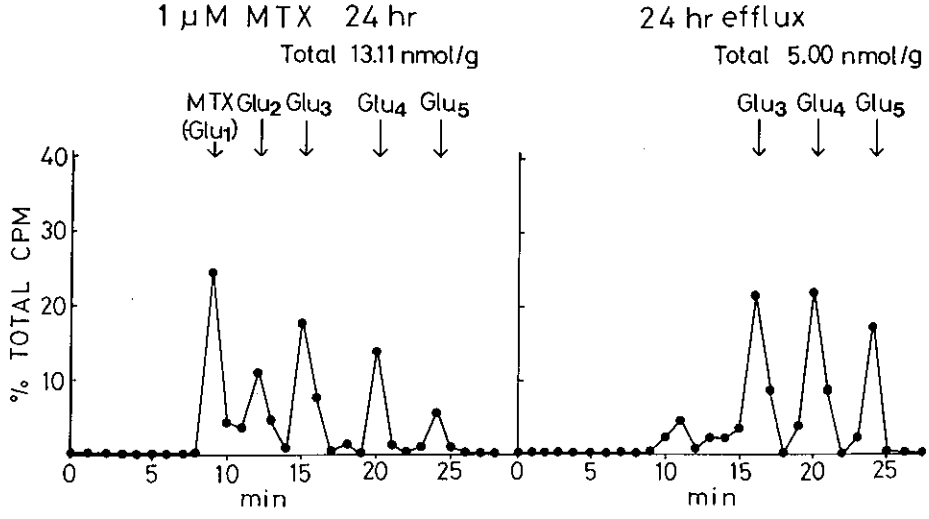

Fig. 1. MTX polyglutamation profile found by HPLC in the parent (sensitive) K562 cells. After a 24-hr incubation with 1μM MTX and after another 24-hr incubation in drug-free medium, cell extracts were analyzed by HPLC to determine the MTX polyglutamation profile. The five peaks represent MTX (-Glu_1), MTX-Glu_2, -Glu_3, -Glu_4 and -Glu_5, respectively.

al.[30] Briefly, cells harvested at the end of each incubation period were washed with ice-cold PBS. After sonication the cell lysate was added to TCA solution at a final concentration of 10% and was left for 5 min on ice. Cellular debris was pelleted by centrifugation at 10,000*g* for 15 min and the cell extract was injected into a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) which had been prepared by prior injection of 2 ml of 100% acetonitrile (CH_3CN, Wako) followed by 5 ml of water. The cartridge was then washed by injecting 5 ml of water, following which MTX and its metabolites were eluted with 2 ml of CH_3CN. The sample was evaporated to dryness under N_2 and resuspended in the initial high-pressure liquid chromatography (HPLC) mobil phase.

MTX and MTX-PGs were separated using an HPLC assay system (Tri-Rotar VI, Japan Spectroscopic Co., Tokyo). The mobile phase was prepared by mixing the effluents from two pumps. Pump A solvent was tetrabutylammonium phosphate (Pic A, Waters Associates), 5m*M* at pH 5.5, while the pump B solvent was 50% CH_3CN. Sample fractions (10–50 μl) containing approximately 2,000 cpm were injected onto a Finepak SIL C8 column (Japan Spectroscopic) and eluted at 1 ml/min by applying gradients of 21–28% CH_3CN and 2.9–2.2m*M* Pic A for 15 min, followed by 28% CH_3CN and 2.2m*M* Pic A for the last 15 min of the separation. The retention times of authentic MTX-Glu_{1-5} (provided by Dr. B. A. Chabner, NCI, Bethesda, MD) were determined by monitoring UV absorbance at 313 nm. One-minute fractions were collected directly into scintillation vials using a fraction collector (LKB 2112, Bromma, Sweden) and assayed for radioactivity by liquid scintillation counting (Aloka 711, Tokyo) (Fig. 1). The protein content was determined by the method of Lowry *et al.*[33]

Results

MTX-resistant Cell Lines Five clones with high resistance to MTX were selected. These cells were essentially the same in size and in other morphological characteristics as the parent K562 cells. Karyotype analysis of these 5 cell lines confirmed that they were human and derived from K562. No significant differences in karyotypes were found among these sublines. The doubling times and sensitivities of the clones to MTX are summarized in Table I. The doubling times of these resistant cell lines were between 27 and 60 hr, which were longer than that of the parent cell line (24 hr). The IC_{50} values of the resistant cells were between 12 and 22 μM MTX, while that of the parent cells was 0.0043μM MTX. The most resistant clone (K562/MTX-1 or -3) was approximately 5,100-fold more resistant to MTX as compared to the parent cells. The resistance to MTX was stable for at least 1

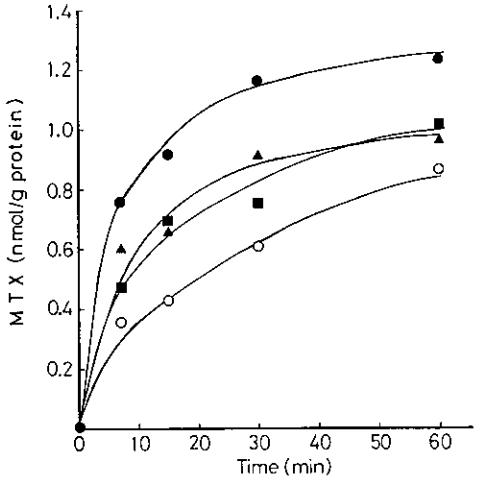

Fig. 2. Uptake of 1μM ^{3}H-MTX by the parent and resistant K562/MTX cells. Data from one of two similar experiments are presented. ●, Parent K562 cells; ○, K562/MTX-3 cells; ▲, K562/MTX-4 cells and ■, K562/MTX-5 cells.

Table I. MTX-resistant Subclones Derived from K562 Cells

Clone	Doubling time (hr)	IC_{50} of MTX (μM)	Index of resistance
Parent K562	20.7±4.4	0.0043±0.0007	1
K562/MTX-1	31.5±3.5	22±1	5100
K562/MTX-2	35.6±2.6	14±5	3300
K562/MTX-3	27.2±4.2	22±1	5100
K562/MTX-4	59.5±1.3	13±3	3000
K562/MTX-5	47.2±3.4	12±3	2800

month in complete medium without MTX in each of the resistant cell lines. The cross resistance to other drugs has not been checked yet.

Short-term Uptake of MTX To elucidate the mechanism of MTX-resistance, short-term (within 1 hr) uptake and accumulation studies were performed. Figure 2 shows the time course of ^{3}H-MTX accumulation by the parent and resistant cell lines during a 60-min exposure to 1μM MTX. Uptake velocity appeared to be linear during the initial 15 min of drug exposure, after which the rate of drug accumulation reached a steady-state level. The initial velocities of MTX uptake in 3 examined resistant clones were 54 to 92 pmol/g protein/min as compared to 110 pmol/g protein/min for the parent line. One-hour accumulations varied from 0.86 to 1.05 nmol/g protein for the resistant lines as compared to 1.22 nmol/g protein for the sensitive parent cell line.

MTX-PG Formation Intracellular accumulations of MTX in the parent cells during 24 hr were 40.56 nmol/g protein and 207.17 nmol/g protein at the concentrations of 1 and 10μM MTX, respectively. In contrast, intracellular levels of MTX in the resistant cell lines were between 36.57 and 89.07 nmol/g protein after 24 hr incubation with 10μM MTX. These levels were almost the same as that of the parent cells incubated with a 10-fold lower concentration (1μM) of MTX. When the resistant cells were incubated with 1μM MTX for 24 hr, total intracellular levels of MTX ranged from 5 to 10 nmol/g protein. Figure 3

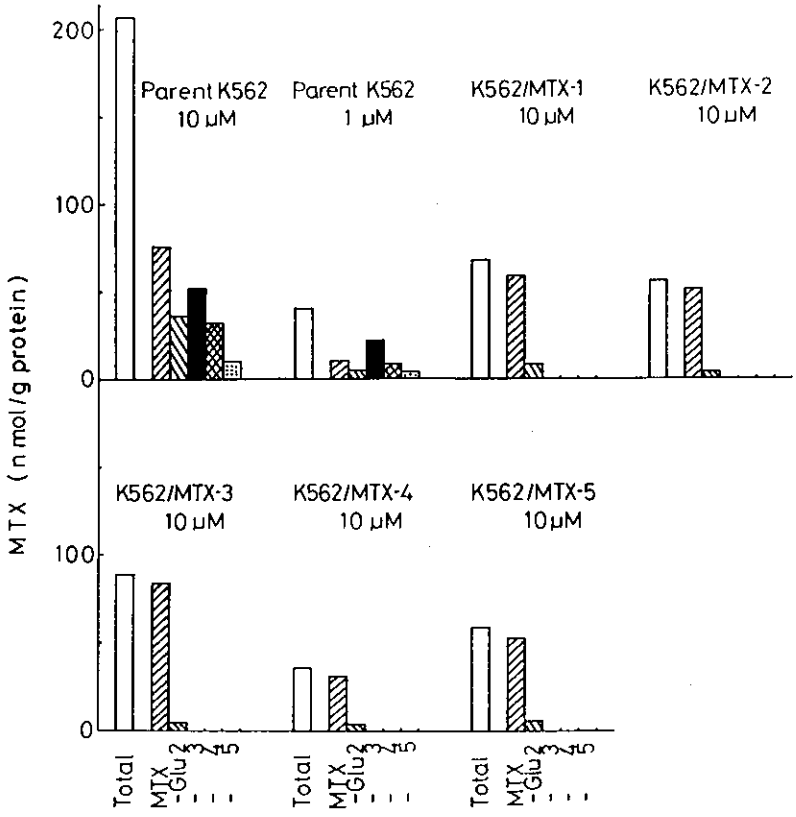

Fig. 3. Total intracellular accumulation of MTX and MTX-PGs during a 24-hr incubation. After a 24-hr incubation with 1 or 10μM MTX, cell extracts were assayed by HPLC for MTX and MTX-PGs. Data from one of three similar experiments are presented. □, Total intracellular MTX; ▨, MTX (-Glu$_1$); ▧, MTX-Glu$_2$; ■, MTX-Glu$_3$; ▩, MTX-Glu$_4$ and ▦, MTX-Glu$_5$.

shows the intracellular total levels of MTX and the MTX-PG profile after 24 hr incubation of the parent cell line and 5 cloned resistant cell lines. The formation of MTX-PG derivatives, MTX-Glu_2 through -Glu_5, was drug concentration-dependent as indicated in the experiments using the parent cells. More interestingly, although the parent cells converted MTX to MTX-PGs with 3 or more glutamyl moieties even at a low concentration ($1\mu M$) of MTX, there was little MTX-PG formation in any of the 5 resistant clones even at a high concentration ($10\mu M$) of MTX. High-molecular-weight MTX-PGs (MTX-Glu_{3-5}) constituted less than 2% of the total intracellular MTX in the resistant cells, while MTX-PGs in the parent cells constituted 46% and 62% in $10\mu M$ and $1\mu M$ MTX, respectively. The absence of MTX-PGs in resistant cells could not be ascribed to decreased transport, in that the intracellular level of MTX-Glu_1 (parent drug) in K562/MTX-3 was equivalent to that in the parent cell line and was only reduced by 1/3 in K562/MTX-1, MTX-2 and MTX-5. In cell line K562/MTX-4, intracellular MTX-Glu_1 was only 1/3 of that in the parent cell line.

We next determined the efflux pattern of MTX-PGs. After 24 hr of efflux, there was no significant difference in the total intracellular MTX levels between the parent cells and the resistant cells. However, the MTX-PG profiles between the parent and the resistant cell lines appeared quite different. As shown in Fig. 4, in the parent cells, little MTX (-Glu_1) and MTX-Glu_2 were retained intracellularly and considerable amounts of MTX-Glu_3, -Glu_4 and -Glu_5 (86% of the total MTX) were preferentially retained. On the other hand, in the resistant cells, no additional MTX-PG formation was demonstrated and most of the intracellular MTX was still retained as a non-

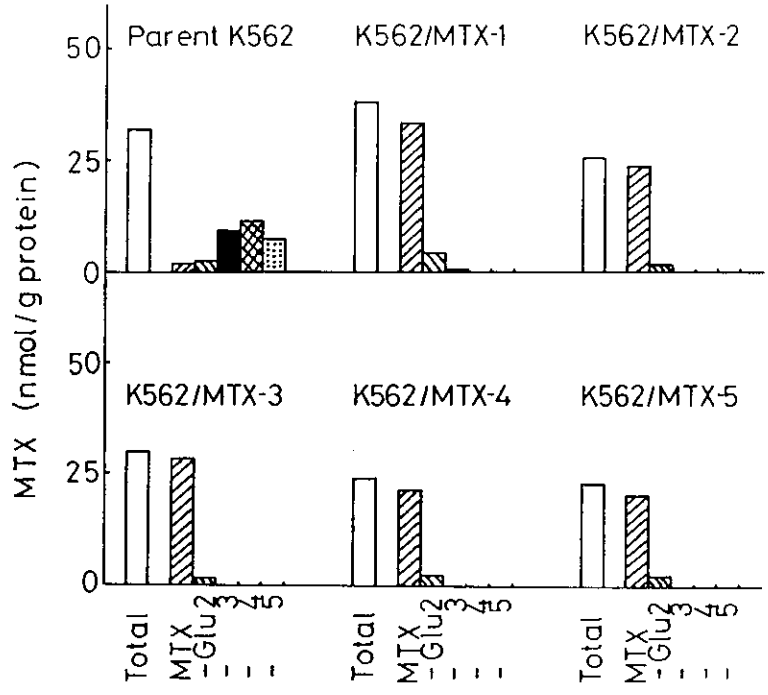

Fig. 4. Efflux of MTX-PGs from the parent (sensitive) and resistant K562/MTX clones. After a 24-hr incubation with $10\mu M$ MTX, cells were washed and incubated for an additional 24 hr in drug-free medium, and then cell extracts were assayed by HPLC for MTX and MTX-PGs. Data from one of three similar experiments are presented. □, Total intracellular MTX; ▨, MTX (-Glu_1); ▧, MTX-Glu_2; ■, MTX-Glu_3; ▩, MTX-Glu_4 and ▩, MTX-Glu_5.

metabolized form ($MTX\text{-}Glu_1$) with a very small amount of $MTX\text{-}Glu_2$. There were no significant differences in the MTX-PG profile among the 5 resistant cell lines.

Discussion

From studies of experimental MTX resistance, a variety of mechanisms have been suggested.[1-3] Our results indicate that an alteration of MTX membrane transport may be one of the mechanisms participating in the drug resistance, because initial drug uptake at a low concentration of MTX was found to be impaired in each of the cell lines, and accumulation of intracellular MTX within 1 hr was only 70–80% of the control. However, the more than 1,000-fold increase of resistance could not be explained by this mechanism since the cells remained resistant in the presence of extracellular levels of $1\mu M$ or greater, which produced high intracellular concentrations of the parent drug.

More interestingly, a decreased polyglutamation of MTX was clearly noted and may contribute to the drug resistance of K562/MTX clones in this study. After 24-hr incubation even with a high concentration (10 μM) of MTX, all 5 resistant clones showed appreciable accumulation of the parent drug but little formation of high-molecular-weight MTX-PGs ($MTX\text{-}Glu_3$, $\text{-}Glu_4$ and $\text{-}Glu_5$). The MTX-sensitive parent K562 cells formed high levels of MTX-PGs which constituted approximately 60% of total intracellular MTX content even at a low concentration ($1\mu M$) of MTX. Marked differences of MTX-PG accumulation between parent and resistant cells were suggested by the efflux profile of MTX-PGs, and may also contribute to the high levels of resistance to MTX. Furthermore, the impairment of MTX-PG formation might be a special mechanism for this high level of MTX resistance which might be observed only under these experimental conditions. Further studies regarding the binding affinity for DHFR, levels of DHFR and TS, and gene analysis are needed to elucidate the precise mechanisms of resistance.

Decreased MTX-PG formation was reported as a novel mechanism of drug resistance in human small-cell lung cancer cell lines[29] and human breast cancer cell lines.[32] In the latter paper, a defect in MTX transport, in addition to the decreased polyglutamation, was found in the MTX-resistant cell line. In addition, an *in vivo* study concerning these mechanisms was reported by Curt *et al.*[31] who investigated the resistance of eight patient-derived small-cell carcinoma cell lines whose resistance was acquired *in vivo*. After a thorough investigation of known causes of MTX resistance, six cell lines were found to be resistant and to have an inability to metabolize MTX to MTX-PGs. The authors also suggested that low TS activity contributed to resistance in two of the cell lines. Furthermore, a limited capacity of purified normal human early myeloid cells to form MTX-PGs, coupled with a high tolerance of the cells to MTX was also investigated.[20]

The most common mechanism of MTX resistance reported in cloned cell lines has been gene amplification of the target enzyme DHFR.[11, 34] We did not measure the enzyme activity or perform DHFR gene analysis of these resistant clones, although chromosomal analysis revealed no presence of homogenous staining regions (HSR) or double minute (DM) chromosomes (data not shown).

Following MTX exposure, *de novo* purine synthesis is markedly inhibited. Recently, Allegra *et al.*[16, 35] developed methods for quantitating intracellular folates using HPLC and applied these techniques to the study of intracellular folate pools after MTX exposure. The results of this study indicate that the inhibition of purine synthesis does not result from the depletion of folate pools, e.g. 10-formyltetrahydrofolate, an important cofactor in purine synthesis, but may result from direct inhibition of the key enzyme, AICAR T'ase, by MTX-PGs formed following drug exposure. Decreased MTX-PG formation, therefore, may contribute to an alternate mechanism of the drug resistance, although we did not measure folate pools or AICAR T'ase activity in this study.

In summary, although these MTX-resistant clones established in this study had a complex pattern of changes, the impairment of MTX-PG formation was striking and, with transport alteration, could contribute to the resistance. The enzyme responsible for this reaction, folylpolyglutamyl synthetase, catalyzes the addition of the glutamate group in

a gamma-linkage to the end carboxyl group of the neighboring folyl glutamate, using ATP as its energy source. Further studies of highly purified preparations of this enzyme are required to understand its regulation, its catalytic mechanism and the structure-activity relationship of potential substrates and inhibitors.

ACKNOWLEDGMENTS

We thank Drs. B. A. Chabner and C. J. Allegra, NCI, for their useful scientific discussions. This work was supported in part by a Grant-in-Aid for Scientific Research (61570447) from the Japanese Ministry of Education, Science and Culture, and a Grant-in-Aid for Cancer Research (62-19) from the Japanese Ministry of Health and Welfare.
(Received June 24, 1988/Accepted October 5, 1988)

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