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Schedule-dependent Synergism and Antagonism between Methotrexate and 6-mercaptopurine in a Human Acute Lymphoblastic Cell Line

Yasuhiko Kano, Miyuki Akutsu, Tadashi Kasahara and Shinobu Sakamoto

We studied the combined cytotoxic effects of methotrexate and 6-mercaptopurine (6-MP) on a human acute lymphoblastic cell line (MOLT-3) *in vitro* according to various schedules. The combined effects were analysed with improved isobologram using the concept of additivity. Simultaneous and continuous exposure (72 h) to these two agents had subadditive to protective effects (antagonism). Partial simultaneous exposure to methotrexate (5 h) and 6-MP (72 h) showed additive to protective effects. Sequential exposure to methotrexate (5 h) followed by 6-MP (72 h) had an additive effect at 0 h and a supra-additive effect (synergism) at 3 h and 19 h intervals. Therefore, it would seem to be better to avoid the simultaneous administration of methotrexate and 6-MP when these two drugs are used in combination. Sequential administration of methotrexate first, followed by 6-MP at short intervals, is recommended.
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INTRODUCTION

METHOTREXATE and 6-mercaptopurine (6-MP) are widely used for the treatment of acute leukaemia and combinations of the two drugs have been employed in many acute lymphoblastic leukaemia maintenance regimens. This suggests that methotrexate and 6-MP have great influence on the outcome of the disease.

6-MP itself is inactive and is converted intracellularly to 6-thioinosine monophosphate (6-TMP) as the first step in its cytotoxic action [1]. This intracellular reaction, which is catalysed by hypoxanthine phosphoribosyltransferase, requires 5-phosphoribosyl-1-pyrophosphate (PRPP). PRPP level has been reported to be an important determinant of 6-MP activity [2, 3]. 6-TMP inhibits several enzymes in the *de novo* purine pathway [1]. 6-TMP is converted to 6-thioguanine monophosphate (6-TGMP), which is further phosphorylated and reduced to 6-thioguanine triphosphate (6-TGTP) and 6-deoxythioguanine triphosphate (6-dTGTP). 6-TGTP and 6-dTGTP are then incorporated into RNA and DNA, respectively [4]. These factors are related to the cytotoxic action of 6-MP. Methotrexate has an antipurine effect which results in the marked elevation of PRPP in the intracellular pool [5, 6]. These facts suggest that combinations of methotrexate and 6-MP would have a synergistic effect, as shown in a methotrexate/5-fluorouracil combination [7]. However, the cytotoxic effects of methotrexate/6-MP combinations have not been studied in detail.

In recent years, much attention has been paid to methods of combining anticancer agents to increase therapeutic effect. The increased therapeutic effect of combination has been based upon data from *in vivo* and *in vitro* studies. Since dose-response survivals or curves are usually not linear, analysis of the data is not easy and most drug combination studies lack an important factor in their analyses of the combinations. Steel and Peckham proposed an improved isobologram method for the analysis of the effects of combined radiotherapy and chemotherapy with non-linear dose-response curves [8]. We have previously reported the use of this method to analyse the effects of drug combinations [9]. Recently, another group has also used this approach [10].

We studied the effects of methotrexate/6-MP combinations on an acute lymphoblastic leukaemia cell line, MOLT-3, *in vitro* according to various schedules and analysed the data with improved isobolograms [8, 9].

MATERIALS AND METHODS

Cell line

A human acute lymphoblastic leukaemia cell line, MOLT-3 [11] was maintained as a suspension culture in culture flasks containing RPMI1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Flow, Rockville, Maryland) and antibiotics (50 μg/ml of gentamicin).

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Cell growth inhibition by combined anticancer agents

Logarithmically growing cells were harvested from the medium and resuspended to a final concentration of 1×10^5 cells/ml of fresh medium with 10% FBS. For the simultaneous and continuous mode of exposure to two drugs, cell suspensions (1 ml) were dispensed into individual wells of a 24-well tissue culture plate with lid (Falcon, Oxnard, California). Each drug solution (10 μl) was added at different concentrations to individual wells containing cell suspensions. The plates were then incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C for 72 h. For partial simultaneous exposure to methotrexate (5 h) and 6-MP (72 h), the cell suspensions were dispensed into tissue culture tubes (Costar, Cambridge, Massachusetts) and exposed to the two drugs at various concentrations at 37°C for 5 h. At the end of this incubation, the cells were washed twice with fresh medium and were then resuspended in the same volume of medium with 10% FBS; the cell suspensions (1 ml) were then dispensed into individual wells of a 24-well plate. 6-MP solution at the same concentrations was added and the cell suspensions were incubated in the same conditions for 72 h. For sequential exposure to methotrexate (5 h) followed by 6-MP (72 h), the cell suspensions were dispensed into tissue culture tubes and exposed to methotrexate for 5 h, washed twice and resuspended in fresh medium with 10% FBS. The cell suspensions (1 ml) were then dispensed into wells and incubated for 0, 3 and 19 h. At the end of this incubation, the cell suspensions were exposed to 6-MP and again incubated for a further 72 h.

Viable cell growth was determined by the trypan blue dye exclusion method. The dose-response curves were plotted on a semilog scale as a percentage of the control, the cell number of which was obtained from samples with no drug exposure, but which had been processed simultaneously. Experiments were repeated at least twice with triplicate samples for each drug concentration.

Isobologram analysis

We studied the effects of methotrexate 6-MP combinations at the point of ID_{80}. The effects of drug combination were analysed by improved isobologram as described previously [9]. Based upon the dose-response curves of methotrexate and 6-MP, three isoeffect curves were drawn (Fig. 1).

Mode I line. When the dose of methotrexate is chosen, an increment in effect remains, which is to be produced by 6-MP. The addition is calculated by taking the increment in doses, starting from zero, that gives log survivals that add up to ID_{80} (heteroaddition).

Mode II (a) line. When the dose of methotrexate is chosen, an increment in effect remains, which is to be produced by 6-MP. The addition is calculated by taking the increment in doses, starting from the point on the dose-response curve of methotrexate where the effect of methotrexate has ended, that gives log survivals that add up to ID_{80} (isoaddition).

Mode II (b) line. Similarly, when the dose of 6-MP is chosen, an increment in effect remains, which is to be produced by methotrexate. The addition is calculated by taking the increment in doses, starting from the point on the dose-response curve of 6-MP where the effect of 6-MP has ended, that gives log survivals that add up to ID_{80} (isoaddition).

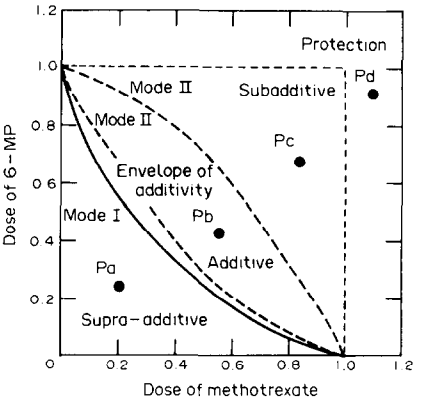

Fig. 1. An envelope of additivity, which is surrounded by mode I and mode II, is constructed from the dose response curves of methotrexate and 6-MP. Combined data points within the envelope show additive interaction. Data points Pa, Pb, Pc and Pd show supra-additivity, additivity, subadditivity and protection, respectively.

With combinations of graded doses of methotrexate (or 6-MP) and a chosen dose of 6-MP (or methotrexate), a single dose-response curve can be drawn. Since we cannot know whether the combined effect of two drugs will be isoadditive, heteroadditive or intermediate, all possibilities should be considered. Therefore, when the data point of a drug combination falls in the area surrounded by three lines (envelope of additivity), this combination is regarded as additive. When the data point falls left of the envelope, the two drugs have supra-additive interaction (synergism). When the data point falls right of the envelope, but within the square, the two drugs have subadditive interaction. When the data point is outside the square, this combination is regarded as protective interaction. Both subadditive and protective interaction are considered as antagonism. In each experiment, dose-response curves of methotrexate, 6-MP and the two drugs were different, but a similar tendency was observed. We chose to present representative dose-response curves and isobolograms.

RESULTS

Dose-response curves obtained with combinations of methotrexate and 6-MP according to various schedules are shown in Fig. 2a–e. Based upon these dose-response curves, isobolograms were made (Fig. 3a–e).

For the simultaneous and continuous exposure to methotrexate and 6-MP, the data points fell on the right side of the envelope (Fig. 3a). Certain data points were in the area of subadditivity and one point was in the area of protection. These observations were interpreted to show that simultaneous exposure to methotrexate and 6-MP produces subadditive to protective interaction. For partial simultaneous exposures of methotrexate (5 h) and 6-MP (72 h), the data points were dispersed in the areas of additivity, subadditivity and protection (Fig. 3b). Sequential exposure to methotrexate and 6-MP produced different interaction. When the cells were exposed to methotrexate for 5 h followed by 6-MP for 72 h, the data points moved from the area of additivity at 0 h (Fig. 3c) into the area of supra-additivity at intervals of 3 and 19 h (Fig. 3d, e).

DISCUSSION

The present study was undertaken in order to determine if scheduling of methotrexate and 6-MP could lead to an increase

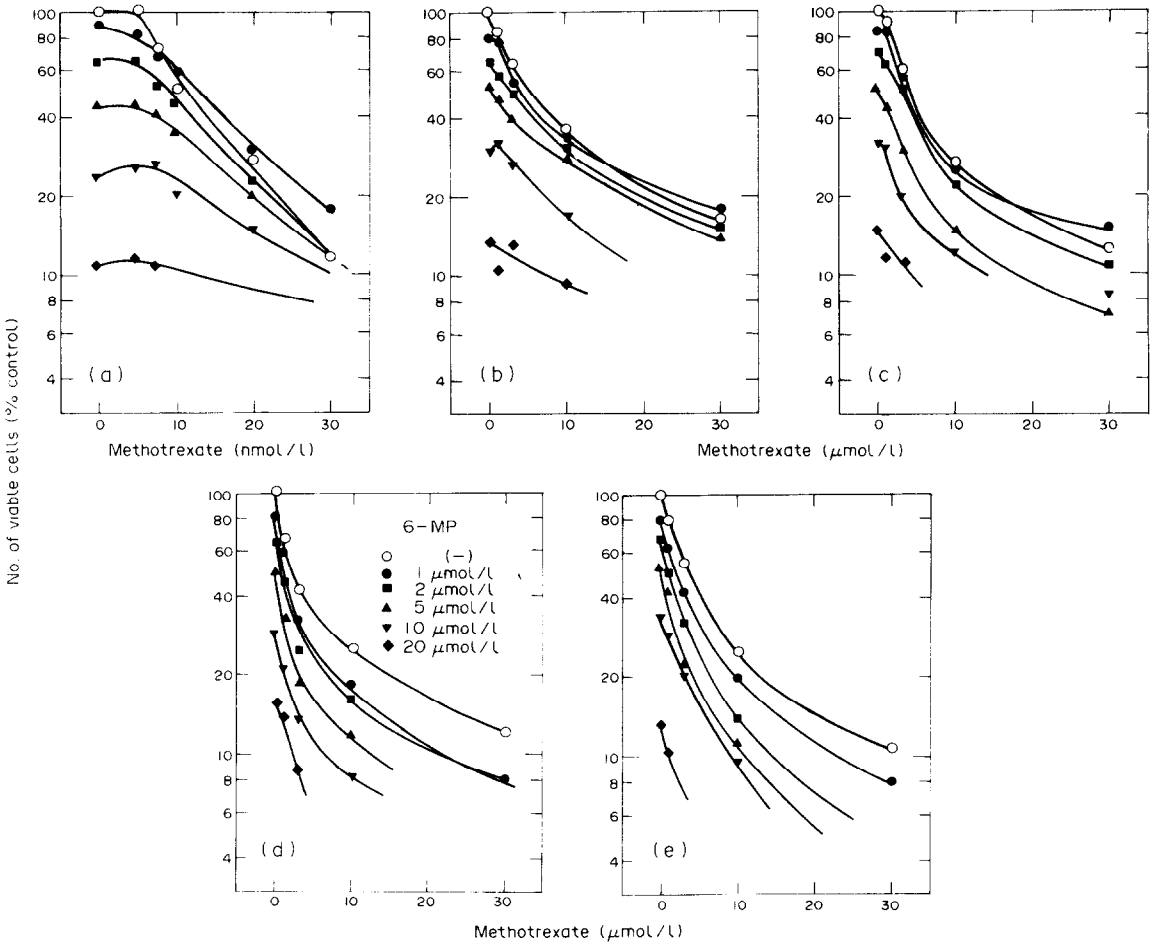

Fig. 2. Dose-response curves in methotrexate 6-MP combinations. (a) Simultaneous and continuous (72 h) exposure to methotrexate and 6-MP; (b) partial simultaneous exposure to methotrexate (5 h) and 6-MP (72 h); (c) short-term exposure to methotrexate (5 h) followed by continuous exposure to 6-MP (72 h) at 0 h; (d) short-term exposure to methotrexate (5 h) followed by continuous exposure to 6-MP (72 h) at a 3 h interval; (e) short-term exposure to methotrexate (5 h) followed by continuous exposure to 6-MP (72 h) at a 19 h interval. 6-MP concentrations (μmol/l) for each symbol are shown at lower left (d).

in therapeutic advantage. Using improved isobolograms, we demonstrated a synergistic cell kill on MOLT-3 when the cells were exposed to methotrexate before 6-MP at 3 h and 19 h intervals. On the other hand, methotrexate and 6-MP had an antagonistic cell kill when the cells were exposed to these two agents simultaneously. This is not consistent with the results of a previous study by De Adreu *et al.* [12]. They reported that both simultaneous and sequential exposure to methotrexate and 6-MP had a synergistic effect on MOLT-4, which was derived from MOLT-3. In their experiments, control cells (no drugs) reached lag phase during culture days since the initial cell number (more than 5×10^5/ml) was too high for 3-day culture. In addition, the analytical methods they used for obtaining the combined effects are unclear.

The mechanism of synergistic interaction in sequential exposure to methotrexate and 6-MP can be explained by the fact that it takes a few hours for methotrexate to enhance cellular PRPP levels, a factor which is important for 6-MP cytotoxicity. On the other hand, the mechanism of the antagonistic effect between methotrexate and 6-MP in simultaneous exposure is unknown. 5-fluorouracil and 6-thioguanine, which require sufficient levels of PRPP to exert their cytotoxicity, also have an antagonistic effect with methotrexate in simultaneous exposure [7, 13]. Since it takes only a few hours for methotrexate to enhance cellular PRPP levels, such a mechanism would also operate in the simultaneous 72 h exposure to methotrexate and 6-MP. 6-MP, 5-fluorouracil and 6-thioguanine may interfere with the cytotoxic effects of methotrexate in some way or, conversely, methotrexate could interfere with the cytotoxic effects of these other drugs.

In this study, the concentration of 6-MP in the medium required for ID_{80} was more than 10 μmol/l, while the plasma level of oral 6-MP is usually less than 1 μmol/l [14]. This may be due to the fact that most 6-MP in the medium is inactivated within 24 h [15]. Interestingly, 6-MP decreases cell kill with increasing drug dose in clonogenic assay using animal cell [15, 16]. The paediatric patients with lower exposure to 6-MP during maintenance therapy for acute lymphoid leukaemia (ALL) showed higher relapse rate [17], suggesting that paradoxical behaviour of 6-MP cannot apply to oral therapy, probably due to low plasma level of 6-MP. In our study, low dose 6-MP (2 and 5 μmol/l) in combination with methotrexate showed definite schedule-dependent antagonism and synergism. Although our studies, based upon 3 days culture cannot evaluate the delayed cytotoxic effects of 6-MP [4, 16], we think that our results could apply to at least the clinical combination of methotrexate and oral 6-MP.

The combination of methotrexate and 6-MP is recommended in several respects. Firstly, some 6-MP-resistant cell lines have collateral sensitivity to methotrexate [18, 19]. Combinations of

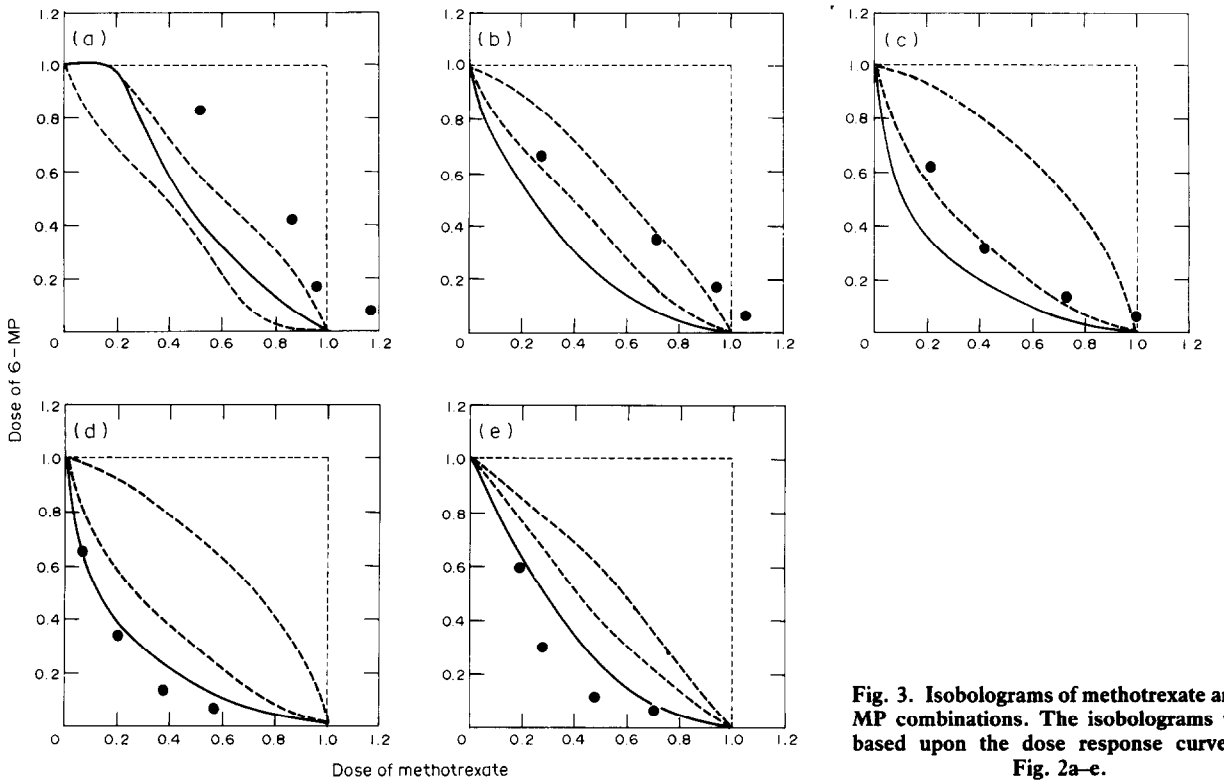

Fig. 3. Isobolograms of methotrexate and 6-MP combinations. The isobolograms were based upon the dose response curves in Fig. 2a–e.

these drugs may overcome 6-MP-resistant clones. Secondly, the circulating level of hypoxanthine is high enough to protect leukaemic cells against the antipurine effect of 6-MP. Methotrexate treatment decreases the hypoxanthine level to almost zero within 24–48 h, suggesting that sequential treatment of methotrexate and 6-MP enhances 6-MP cytotoxicity [20]. Thirdly, methotrexate and allopurinol are inhibitors of xanthine oxidase, which oxidises 6-MP to the inactive metabolite 6-thiouric acid [21]. Absorption of oral 6-MP is known to be poor and highly variable [22] and large individual variations in the pharmacokinetics of 6-MP may be one of the important factors of therapeutic failure in ALL [17]. This variation may be primarily a result of catabolic metabolism by xanthine oxidase. Balis *et al.* reported that oral 6-MP in combination with methotrexate increased both mean plasma concentration and area under the curve (AUC) of 6-MP levels in patients with ALL when compared with levels achieved with 6-MP alone [14]. However, simultaneous administration of methotrexate and 6-MP has an antagonistic effect on tumour cell kill, suggesting that this schedule is not suitable for combination. Balis *et al.* did not study the pharmacokinetics of 6-MP after sequential administration of methotrexate and 6-MP, but methotrexate followed by 6-MP at short intervals seems to be more effective for the inhibition of xanthine oxidase which results in enhancement of plasma 6-MP level. Further studies are needed to define the pharmacokinetics of 6-MP in this sequential schedule.

In summary, although the combination of methotrexate and 6-MP seems favourable in several respects, our data suggest that simultaneous administration of methotrexate and 6-MP is less effective for cytokilling than expected. Sequential administration of methotrexate and 6-MP at short intervals may be important in optimising the effectiveness of combined treatment. Although there is a gap between *in vitro* experiments and clinical trials, our data will be helpful in the planning of new protocols containing methotrexate and 6-MP.

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Comparison of the Effects of the Irreversible Aromatase Inhibitor Exemestane with Atamestane and MDL 18962 in Rats with DMBA-induced Mammary Tumours

Tiziana Zaccheo, Donata Giudici, Giorgio Ornati, Achille Panzeri and Enrico di Salle

The antitumour activity of the steroidal aromatase inhibitors exemestane (FCE 24304), MDL 18962 and atamestane (SH 489) was evaluated on 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumours in rats. The compounds were given subcutaneously at daily doses of 10 and 50 mg/kg for 4 weeks. Exemestane was also given orally, at daily doses of 100 and 200 mg/kg. Subcutaneous exemestane induced 30% (10 mg/kg) and 73% (50 mg/kg) regressions of established tumours and strongly reduced the appearance of new tumours. Conversely, atamestane, MDL 18962 and oral exemestane did not affect growth of established tumours nor influenced the appearance of new neoplasms. Aromatase activity of ovarian microsomes (OAA) was reduced by 85%–93% after subcutaneous exemestane and by 25%–59% after MDL 18962, and was unaffected after atamestane. Oral exemestane caused a reduction in OAA of 72%–74%. Serum luteinising hormone (LH) levels were reduced at both the subcutaneous doses of exemestane and at the higher dose of MDL 18962. Atamestane caused an increase in LH levels, while no effect was observed with oral exemestane. The LH-lowering effect of subcutaneous exemestane, the less marked effect of MDL 18962, and the ineffectiveness of oral exemestane were also observed after 10 days of treatment in ovariectomised rats. The antigonadotrophic effect of subcutaneous exemestane, which is probably due to its slight androgenic effect, could contribute to its antitumour activity in the DMBA tumour model in intact rats, through a counteraction of the negative feedback of oestrogens on gonadotropin secretion.
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INTRODUCTION

MUCH INTEREST in hormonal treatment strategies of breast cancer has recently focused on the blockade of oestrogen biosynthesis as a mean for inducing regressions of hormone-dependent tumours [1]. To this end, specific inhibitors of the enzyme aromatase—i.e. the enzyme system catalysing the conversion of androgens into oestrogens—have been developed. Besides the development of reversible non-steroidal aromatase inhibitors, resembling the mechanism of action of the pioneer drug aminoglutethimide, attention has also been directed towards the development of irreversible steroidal aromatase inhibitors, chemically related to the natural substrate androstenedione [1]. 4-hydroxyandrostenedione (4-OHA) may be regarded as the prototype compound of this class [1, 2]. In