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Growth kinetics of L1210 leukemic cells exposed to different concentration courses of methotrexate in vitro

Stefan Gimmel, H. Rainer Maurer

Institute of Pharmacy, Free University of Berlin, Berlin, Germany

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Abstract. At present, pharmacokinetic aspects in existing in vitro assays for cytotoxic drug screening are considered only insufficiently. Using a new microperfusion assay, we integrated the peak plasma concentration (C_{max}), the time of peak concentration (t_{max}), the absorption rate, and the elimination rate following methotrexate (MTX) treatment of L1210 leukemic cells in vitro. The effects of different MTX concentration courses with constant exposure doses (area under the concentration-time curve) were checked by combining the microperfusion assay with the clonogenic assay in agar-containing glass capillaries. We found that the concentration profiles obtained in the ultrafiltration-flat chamber (an essential part of the microperfusion system) can be described by the Bateman function. Thus, the flat chamber might be comprehended as a one-compartmental system with first-order absorption kinetics. We found that the colony-inhibition kinetics of L1210 cells obviously depended on the MTX exposure profile. Continuous cell-growth inhibition was obtained by one concentration profile that offered a compromise between all pharmacokinetic parameters. Our results correlated with the known pharmacodynamic activities of MTX and showed the relevance of different concentration courses to the cytotoxic effect of the drug. We suppose that the growth-inhibition kinetics of unknown, potential anticancer drugs can be also interpreted in similar ways.

Key words: In vitro microperfusion system – L1210 cells – Methotrexate – Pharmacokinetic parameters

Introduction

Comparisons of the in vitro cytotoxicity of anticancer drugs with that in vivo are problematic, because pharmacokinetic courses are seldom considered or rarely integrated in vitro. Nevertheless, to obtain quantitative relationships, models have been developed relating cytotoxic effects to drug concentrations, exposure times, or exposure doses ($c \times t$) [1, 19]. The $c \times t$ product in vitro is set equal to the integral of the drug concentration-time curve (AUC, area under the curve) in vivo. Obviously, different concentration profiles in vivo may have the same AUC, although the peak plasma concentration (C_{max}), time of peak concentration (t_{max}), absorption rate (k_a), or elimination rate (k_e) can differ widely [3]. However, the efficacy of anticancer drug treatment varies in spite of a constant AUC if the pharmacokinetic parameters change [11]. Also the side effects of cytotoxic drugs vary, as has been reported for methotrexate (MTX) [17]. Thus, it is important that investigators obtain more data on the behavior of tumor cells exposed to drugs under naturally occurring conditions in anticipation of time-consuming and expensive animal studies.

To this end, we recently developed a microperfusion system for exposure of tumor cells to anticancer drugs so as to integrate pharmacokinetic parameters in cytotoxicity assays [6]. After validation of the method [5], we studied the growth-inhibition kinetics of L1210 cells exposed to different concentration courses of MTX. Exposure doses were kept constant over the exposure period after "bolus injection", Pharmacokinetic parameters were varied by changing the "pumping rate of the mobile phase" (medium) and the "bolus drug dose", whereby the colony growth of L1210 leukemic cells was dependent on exposure courses. For MTX, a time-dependent mode of action has been established [5], but the C_{max} of the concentration profile should not be neglected. According to evaluations in microbiology studies, we calculated the AUC above the minimal inhibitory concentration (MIC), the time above the MIC, and the inhibitory quotient [4, 15] and evaluated these data with reference to the pharmacodynamics of MTX.

Materials and methods

Drug determination. MTX was purchased from Sigma (Deisenhofen, Germany) and the stock solution (450 μg/ml) in 12 m*M* hydrochloric acid was stored at –20° C. MTX in RPMI 1640 medium without phenol

Correspondence to: H. Rainer Maurer, Institut für Pharmazie, Frie Universität Berlin, Kelchstrasse 31, D-12169 Berlin, Germany

red (Biochrom, Berlin, Germany) was assayed by a simple high-performance liquid chromatographic (HPLC) column-switching method as previously described [5].

Cells and cytotoxicity assay. L1210 murine leukemia cells were obtained from ASTA Medica Laboratories (Frankfurt/Main, Germany). Cells were maintained in RPMI 1640 medium supplemented with 10% horse serum and a 0.2% mixture of penicillin (50,000 IU/ml) and streptomycin (0.05 g/l; Boehringer, Mannheim, Germany). Incubation in an atmosphere containing 5% CO_2 at 37° C and a relative humidity of ≤100% led to a cell population-doubling time of 12 h. For cytotoxicity assay, cells were cultured in suspension in an ultrafiltration-flat chamber (Schütt, Göttingen, Germany) [9, 10] as described elsewhere [6]. A YM30 ultrafiltration membrane (Amicon, Witten, Germany) was used to retain cells in the flat chamber. The stirring speed of cells was adjusted to 110 rpm. A defined quantity of MTX was injected through the "lock" of the microperfusion system. By changing the pumping rate, we created different courses of MTX concentration in the flat chamber. After incubation periods of 10, 90, 180, and 300 min, 100-µl samples were collected. Cells were centrifuged, washed twice, and resuspended in RPMI 1640 medium. Aliquots of 40 µl were tested by the colony-forming assay (CFA) [6], which was executed in agar-containing capillaries as described by Maurer and Echarti [12]. Calculations for the CFA and statistical evaluation were carried out as previously reported [6].

Characterization of drug concentration courses. A defined, always identical MTX dose (4.5 µg/ml) was pumped through the lock into the flat chamber. After passing the chamber, fractions of MTX-medium mixtures were collected (500 µl), and the MTX concentration in each fraction was determined by HPLC. Pumping rates were adjusted to 0.01, 0.02, 0.04, 0.1, 0.13, and 0.17 ml/min. Concentration-time profiles were checked in duplicate in relation to each pumping rate. The computer program SIPHAR 4.0 (Simed, Creteil, France) was used to evaluate the pharmacokinetic parameters (C_{max}, t_{max}, k_e, k_a). Concentration courses followed first-order kinetics and, hence, could be described by the Bateman function (see Eq. 1). Drug absorption and elimination rates in the flat chamber were computed and used to calculate different drug kinetics with an identical exposure dose.

Calculation of the applied drug dose. Concentration courses in the flat chamber followed the Bateman function:

$$C_{pt} = \frac{f \times D}{V_d} \times \frac{k_a}{k_a - k_e} \times (e^{-k_e t} - e^{-k_a t}), \quad (1)$$

where C_{pt} is the concentration at time t, f is the absorbed part of the drug dose, D is the drug dose, V_d is the volume of distribution, k_a is the absorption constant, k_e is the elimination constant, and t is time. Component f could be neglected, because the absorbed part of the drug dose in the flat chamber is 100% (f = 1). The AUC for the optimized exposure period of 300 min can be described by the following equation:

$$AUC_{0-300\ min} = \int_0^{300} C_p(t)dt. \quad (2)$$

Insertion of Eq. 1 into Eq. 2 gives the following equation:

$$AUC_{0-300\ min} = \frac{D}{V_d} \times \frac{k_a}{k_a - k_e} \times \int_0^{300} (e^{-k_e t} - e^{-k_a t})\ dt. \quad (3)$$

Integration of Eq. 3 leads to the following equation:

$$AUC_{0-300\ min} = \frac{D}{V_d} \times \frac{k_a}{k_a - k_e} \times \left[\frac{1}{k_a} \times (e^{-300k_a} - 1) - \frac{1}{k_e} \times (e^{-300k_e} - 1)\right] \quad (4)$$

By solving Eq. 4, we obtain the drug dose that has to be put into the flat chamber. Extension with $k_a \times k_e$ leads to the following equation:

$$D = AUC_{0-300\ min} \times V_d \times \frac{(k_a - k_e)\ k_e}{k_e \times (e^{-300k_a} - 1) - k_a \times (e^{-300k_e} - 1)}. \quad (5)$$

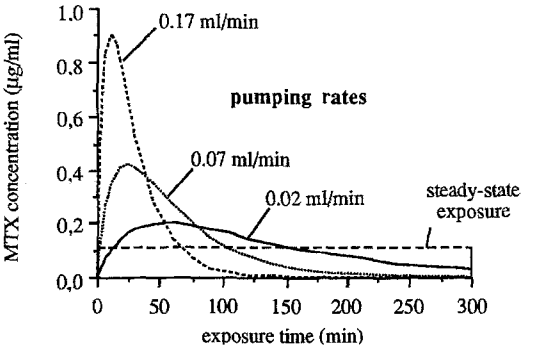

Fig. 1. Courses of MTX concentrations in the flat chamber: constant exposure dose over 5 h (AUC = 33 min µg ml^{-1})

Table 1. Pharmacokinetic parameters and applied drug dose: influences of MTX absorption and elimination on L1210 cells in the flat chamber using constant exposure doses[a]

	Pumping rate [ml/min]	k_a [h^{-1}]	k_e [h^{-1}]	t_{max} [min]	C_{max} [µg/ml]	Applied drug dose [µg]
Steady-state exposure	0.2	–	–	–	(IC_{50}) 0.11	
Non-steady-state exposure	0.02	1.93	0.49	57.6	0.19	0.99
	0.07	5.62	1.16	21.0	0.43	2.11
	0.17	13.00	2.52	9.6	0.93	4.55

[a] $AUC_{0-300\ min}$ = 33 min µg ml^{-1}

Results

We supposed that pharmacokinetic influences on the cytotoxic activity of MTX should be observed within relative small ranges of concentration. Low MTX exposure doses (AUC) should have no effect, and a high AUC might lead to an increased assimilation of the cytotoxicity of different concentration courses. Our tests were carried out using the IC_{50} value (0.11 µg/ml) for steady-state exposure of L1210 cells to MTX over a 300-min period as the exposure dose ($AUC_{0-300\ min}$), which corresponded to a $(c \times t)_{50}$ value of 33 min µg ml^{-1}. The $(c \times t)_{50}$ was defined as the exposure dose that reduces the colony growth to a half-maximal level [5]. In another series of experiments the $AUC_{0-300\ min}$ was doubled. Absorption and elimination rates were varied by changing the pumping rate, and drug doses were calculated for each kinetic with Eq. 5 (Table 1). We used three different pumping rates (0.02, 0.07, and 0.17 ml/min) – resulting in different pharmacokinetic courses – to determine the colony-growth kinetics of L1210 cells (Fig. 1). Different growth-inhibition kinetics of L1210 cells were obtained after their exposure to three concentration-time courses with an identical AUC (Fig. 2). Steady-state exposure reduced the colony growth to 48.3% ± 6.1% of the control value after a lag time had passed. This result was expected because the $(c \times t)_{50}$ value was used.

No cytotoxicity was observed at a pumping rate of 0.02 ml/min with the longest t_{max} and the lowest C_{max}. The reduction in colony numbers to 80% of the control value after 300 min exposure to MTX has no significance in re-

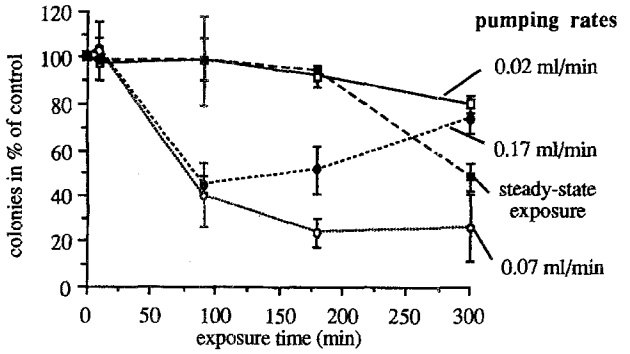

Fig. 2. Colony growth of L1210 cells in the flat chamber: influence of different MTX concentration courses using a constant exposure dose [$AUC_{0-300\ min} = (cxt)_{50} = 30$ min μg ml^{-1}; mean values ± SD; $n = 3$]

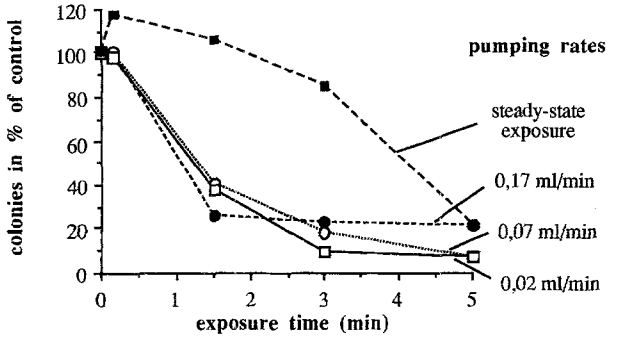

Fig. 3. Colony growth of L1210 cells in the flat chamber: influence of different MTX concentration courses using a constant but doubled exposure dose [$AUC_{0-300\ min} = 2\times (cxt)_{50} = 66$ min μg ml^{-1}; mean values; $n = 2$]

lation to the decrease in colony growth observed in the microperfusion system in the absence of cytotoxic drug (89% after 300 min), as we have recently described [6]. The "fast kinetic" obtained with a pumping rate of 0.17 ml/min (high C_{max}, short t_{max}) led to a significant decrease in colony growth during the first 3-h period to up to 45% of the control value ($P < 0.01$). Subsequently, inhibition diminished again, and after 300 min a colony growth of 74.0% ± 8.0% was obtained, which differed significantly from that observed after 180 min (51.4% ± 13.2%; $P < 0.05$). Only at a pumping rate of 0.07 ml/min did we observe a growth-inhibition kinetic showing a sustained cytotoxic effect over the exposure period. The colony growth decreased to 40.3% ± 17.1% (after 90 min), 23.3% ± 7.6% (after 180 min), and 26.1% ± 17.6% (after 300 min). Destruction of the cell-membrane integrity was not shown by the trypane blue dye-exclusion method [14]. In all cases, viability was greater than 95%.

By doubling the exposure dose (AUC), we obtained identical growth-inhibition kinetics (Fig. 3). In comparison with the influence of steady-state exposure, all three concentration courses led to a quicker reduction of colony growth during the first 90-min period. There was a trend of the "slower" kinetics (0.02 and 0.07 ml/min) toward continuous inhibition, whereas at the pumping rate of 0.17 ml/min, the colony growth after 90 min remained constant during exposure. Moreover, we calculated the AUC above the IC_{50}, the inhibitory quotient (C_{max}/IC_{50}), and the time above the IC_{50} for either concentration profile [4, 15] (Table 2). Altogether, the results verified the principle of dose-finding that we chose. The differences between the growth-inhibition kinetics that were obtained by the application of $(c \times t)_{50}$ were equalized by doubling of the exposure dose.

Discussion

Recently we developed and validated the microperfusion method to integrate pharmacokinetic parameters in screening assays for anticancer drugs [5, 6]. Two applications of the method are imaginable: (1) screening of new potential drugs and (2) screening of known cytotoxic drugs for an individual patient's tumor. As we are interested in the former and since pharmacokinetic data are seldom available at this stage of drug screening, a method had to be set to realize kinetics tests. Thus, dose-finding and a choice of kinetic courses played a decisive role. For determination of the influence of drug absorption and elimination on cell proliferation, the exposure dose ($c \times t$ = AUC) was kept constant over a period of 5 h. Concentration courses appearing in the flat chamber can be described by the Bateman function. Thus, the flat chamber might be comprehended as a one-compartmental system with first-order absorption kinetics.

A cytotoxic agent exerts an inhibitory effect only if its concentration-time profile exceeds the minimal inhibitory concentration (MIC). Consequently, substances with the same exposure dose but different pharmacokinetic parameters will be effective only if the velocity of absorption is fast enough [3]. The effective exposure dose would better be defined as the AUC above the MIC (Fig. 4). In microbiology studies, either the AUC above the MIC is related to the area under the inhibitory curve [15] or an inhibitory quotient is

Table 2. Colony growth in the flat chamber related to the different MTX concentration-time-profile parameters above the IC_{50}

	MTX concentration profiles				Cytotoxicity: Colony growth (% of control)		
Pumping rate (ml/min)	AUC (min μg ml^{-1})	AUC above IC_{50} (min μg ml^{-1})	Inhibitory quotient C_{max}/IC_{50}	Time above IC_{50} (min)	90 min	180 min	300 min
0.02	33	7.2	1.73	142.8	98.7%	91.9%	79.9%
0.07	33	16.2	3.91	100.8	40.3%	23.3%	26.1%
0.17	33	23.4	8.45	64.8	45.0%	51.4%	74.1%

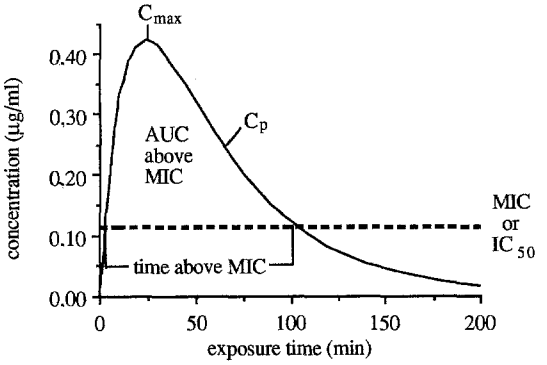

Fig. 4. Alteration of exposure dose (AUC), time, and concentration by consideration of the minimal inhibitory concentration (MIC) or IC_{50}. C_{max}, Peak concentration; C_p, course of concentration in the flat chamber [inhibitory quotient = C_{max}/MIC or C_{max}/IC_{50}]

calculated from the relationship between the peak concentration (C_{max}) and the MIC [4], which has been found to correlate with the clinical response [13].

In clonogenic assays the determination of MIC is very difficult. As regards cell-cycle-specific drugs, it has to be borne in mind that in tests using mammalian cells, great deviations in MIC might occur due to the heterogeneity of cell-cycle-phase distribution. In these cases the IC_{50} offers an approach to determining the MIC. Thus, we used the AUC above the IC_{50}, the inhibitory quotient, and the time above the IC_{50} to evaluate our results (Table 2).

Comparisons of the kinetics obtained with pumping rates of 0.07 and 0.17 ml/min showed the time-dependent effect of MTX, used as a known [17] and – under exposure conditions – stable [5] cytotoxic drug. At 0.07 ml/min, a continuous cytotoxic effect was obtained with a longer exposure time above the IC_{50}. On the other hand, with an increasing inhibitory quotient at a pumping rate of 0.17 ml/min, we might normally expect an increase in cytotoxicity. Three different time-dependent phenomena may be responsible for this result: (1) the time dependence of MTX influx into the cells, (2) the time dependence of the formation of MTX-polyglutamate, or (3) the time dependence of the MTX and MTX-polyglutamate reaction with the target enzyme dihydrofolate reductase. The latter can be neglected because of its high affinity for the target ($Ki \approx 10^{-12} M$) [20]. MTX influx into L1210 cells follows a linear kinetic pattern until intracellular steady-state concentrations are attained (in 20 min [2] or 40–50 min [18]). Comparing these data with our results, we suppose that the extracellular concentration is never attained intracellularly by the "fast" kinetic (0.17 ml/min; t_{max}, 9.6 min), whereas at a pumping rate of 0.07 ml/min (t_{max}, 21 min), MTX uptake occurred at approximately the same velocity as did the increase in extracellular concentration in the flat chamber. The quantity of MTX-polyglutamate formed by the cells [17] is dependent on the cellular MTX concentration. Hence, it may be suggested that differences in the kinetic effects result from MTX influx into the cells. The efflux of MTX – depending on the adenosine triphosphate (ATP) content of cells [16] – occurs via the same transport system as does the influx [8] and, in general, requires less time for the drug levels to adjust to a nonexchangeable intracellular concentration [2, 18]. Under "fast" kinetics, cells are not exposed at all to MTX for a while; consequently, the gradient between intra- and extracellular MTX concentrations may be greater than that observed under "slow" kinetics. It follows that MTX can leave the cells to a large extent. This may explain the data on the exposure times of 90 and 300 min. At a 0.07-ml/min pumping rate, MTX-polyglutamate formation could contribute to a growth inhibition maintained between 90 and 300 min.

At a pumping rate of 0.02 ml/min, no cytotoxic effect was seen in spite of the longest exposure time above the IC_{50}. In this case, the peak concentration and the inhibitory quotient might not have been high enough. Ellner and Neu [4] have proposed an inhibitory quotient of at least 4:8 as a general rule for antibiotic treatment. Furthermore, Goldman [7] has reported that the intracellular MTX concentration required for maximal cytotoxic effect must exceed the binding capacity of dihydrofolate reductase, which corresponds to an intracellular level of 1×10^{-6} *M* MTX. Supposing that the intracellular concentration is equal to the extracellular concentration, an inhibitory quotient of 4.1 can be calculated for this MTX level. This quotient corresponds well to Ellner and Neu's proposal. Increasing the exposure dose led to an assimilation of growth-inhibition kinetics as described above. This result verified the principle of in vitro dose-finding. We do not claim that in vivo dose-finding studies can be performed with our in vitro method under the conditions described. However, pharmakokinetic data obtained in vivo remain to be compared with in vitro data for the further exploitation of potential correlations.

In conclusion, our results demonstrate that the microperfusion method yields data that are relevant not only to cell-cycle-phase specificity and nonspecificity [5] but also to other pharmacodynamic effects (e.g., influx into and efflux out of the cells, intracellular concentration). We suppose that the growth-inhibition kinetics of unknown, potential cytotoxic drugs would allow similar possibilities of interpretation.

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