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ORIGINAL RESEARCH

Genotoxicity of pemetrexed in human peripheral blood lymphocytes

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Abstract Pemetrexed (PMX) is an antineoplastic antifolate used in the treatment of non-small cell lung cancer, mesothelioma and several types of neoplasms. Its toxicity in tumor cells has been linked with the potent inhibition of thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyl transferase, and subsequent depletion of both purine and pyrimidine nucleotides. However, cytogenetic toxicity of PMX in non-diseased cells has not been adequately studied; despite the increasing data on the DNA-damaging potential of antineoplastic agents on normal cells. In the present study, the genotoxic potential of PMX was evaluated in peripheral blood lymphocytes obtained from healthy human subjects using chromosome aberration (CA), sister chromatid exchange (SCE) and micronucleus (MN) assays as the cytogenetic damage markers. Human peripheral blood lymphocytes were exposed to four different concentrations (25, 50, 75 and 100 μg/mL) of PMX for 24- and 48-h treatment periods. PMX significantly increased the formation of CA in 24-h treatment, but not in 48-h treatment. PMX did not increase the mean SCE frequency in 24- and 48-h treatment periods; however, there was a striking increase (although not statistically significant, $p > 0.05$) in the number of SCEs at 25 μg/mL (24- and 48-h treatment) and 50 μg/mL (24-h treatment) due to an increase of SCE at the single-cell level. Interestingly, PMX did not induce MN formation in either 24- or 48-h treatment periods. PMX strongly decreased the mitotic index (MI), proliferation index (PI) and nuclear division index (NDI) in 24- and 48-h treatment periods. Our results suggest that PMX has a potent cytotoxic effect against human peripheral blood lymphocytes at concentrations which are reached in vivo in the blood plasma.

Keywords Chromosome aberration · Micronucleus · Sister chromatid exchange · Cytotoxicity · Pemetrexed

Introduction

Pemetrexed (PMX) is a folic acid analogue with a mode of action responsible for disrupting folate-dependent biosynthetic cycles required for both purine and pyrimidine synthesis. PMX potently exerts clastogenic and cytotoxic effects on tumor cells by inhibiting thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT) (Shih et al. 1998). The inhibition of TS results in depletion of dTTP pools and promotes misincorporation of deoxyuridine triphosphate (dUTP) into newly synthesized DNA, which then leads to the formation of chromosome breakage (Blount et al. 1997; Tonkinson et al. 1997). In

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antifolate-treated normal cells, the inhibition of the crucial enzymes along the de novo pathway of purine and pyrimidine synthesis have been shown to cause gross chromosomal abnormalities and transmissible chromosomal damage (Choudhury et al. 2001; Chow et al. 1998).

PMX [N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid] is used against a wide variety of neoplasms including lymphoma, pancreatic, liver, lung, breast, ductal, prostate, genital neoplasms, gestational trophoblastic tumour and mesothelioma (Dalkic et al. 2010; Shih et al. 1998). It has been previously argued that polyglutamated derivatives of PMX are selectively accumulated in susceptible tumor cells at substantially higher levels than in non-diseased progenitor cells (Chattopadhyay et al. 2007). However, PMX treatment in cancer patients has been frequently associated with low blood cell counts. Signs of PMX toxicity primarily include myelosupression and neutropenia (Kao et al. 2010; Smit et al. 2003).

The small number of genotoxicity studies on antifolates, especially with methotrexate (MTX) and aminopterin (APT), has indicated that these compounds can induce both short and long term DNA damage in several in vitro and in vivo test systems. The genotoxic damage was associated with chromosome and chromatid aberrations, and micronucleus formation in human peripheral blood, in in vivo animal studies and in vitro cell culture assays (Chow et al. 1998; Hittelman 1973; Jensen and Nyfors 1979; Kasahara et al. 1992a).

Although PMX has been investigated in several in vitro tumor cell lines to gain mechanistic insight into its cytotoxic effect, there still exists a lack of appropriate in vitro model for the evaluation of the genotoxicity of PMX on normal cells. Therefore, the genotoxic potential of PMX was investigated in cultured human peripheral blood lymphocytes. Chromosome aberration (CA), sister chromatid exchange (SCE) and micronucleus (MN) were used as the cytogenetic damage markers. Besides, cytotoxicity of PMX was evaluated using the mitotic index (MI), the proliferation index (PI) and the nuclear division index (NDI).

Materials and methods

Commercially available pemetrexed disodium (PMX) (ALIMTA, manufactured by Eli Lilly, Indianapolis, IN, USA) was used as the test substance for the in vitro tests. The chemical formula (Fig. 1) and properties of PMX are shown as follows:

CAS number: 150399-23-8
Linear formula: $C_{20}H_{21}N_5O_6$
Molecular weight: 427.410 g/mol

Concentration selection

The test concentrations were chosen as 25, 50, 75, and 100 μg/mL (roughly 2,25–9 times less than the maximum plasma level (225 μg/mL) achieved in patients receiving this drug) based on the top concentration (100 μg/mL) that resulted a ≥50 % (LD_{50}) reduction in the mitotic index (MI).

In vitro chromosome aberration and sister chromatid exchange assay

The methods of Evans (1984) and Perry and Thomson (1984) were followed for preparation of the CA and the SCE tests with minor modifications. This study was conducted according to the IPCS guidelines (Albertini et al. 2000). Whole blood (0.2 mL) from four healthy donors (two male and two female, nonsmokers, age: 22–25), was added to 2.5 mL PB-MAX (GIBCO—Life Technologies, Carlsbad, CA, USA) chromosome medium, and a final concentration of 10 μg/mL of BrdU was added to the medium just after setting up the blood cultures. Cultures were incubated at 37 °C for 72 h. The cells were treated with 25, 50, 75, and 100 μg/mL concentrations of PMX dissolved in sterile bidistilled water, for 24 h (PMX was added 48 h after initiating the culture) and 48 h (PMX was added 24 h after initiating the culture), respectively. A negative

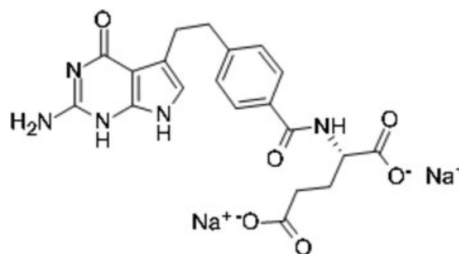

Fig. 1 Chemical structure of disodium pemetrexed

control and a positive control (Mitomycin-C, 0.25 μg/mL, Sigma M-05030 (Sigma, St. Louis, MO, USA) were also used. The cells were exposed to colchicine (0.06 μg/mL, Sigma C9754) 2 h before harvesting. At the end of the incubation, cells were centrifuged at 2,000 rpm for 5 min. Then, the cells were treated with 0.4 % KCl (37 °C) as the hypotonic solution and methanol: glacial acetic acid (3:1) as the fixative (at room temperature 22 °C ± 1 °C, fixative treatments were repeated three times). The cells were centrifuged at 1,200 rpm for 10 min after each fixative treatment. The staining of the air-dried slides was performed following the standard methods using 5 % Giemsa stain for CA and the modified fluorescence plus Giemsa method for SCE (Speit and Haupter 1985). To obtain sister chromatid differentiation (SCD), the slides were irradiated with 30 W, 254 nm UV lamp at 15 cm distance in Sorensen buffer for 30 min, then incubated with 1× SSC (standard saline citrate) at 60 °C for 50 min and stained with 5 % Giemsa prepared with Sorensen buffer. The percentage of cells showing structural and/or numerical chromosome alterations were obtained by calculating the percentage of the aberrant metaphases from each concentration and treatment period. The CA was classified according to the ISCN (International System for Human Cytogenetic Nomenclature) (Paz-y-Miño et al. 2002). CAs were evaluated in 100 well-spread metaphases per donor (totally 400 metaphases per concentration). Gaps were not evaluated as CA (Mace et al. 1978). CAs were classified as structural and numerical aberrations. Structural CAs consisted of the chromatid type (breaks and exchanges) and the chromosome type (breaks, fragments, sister unions, dicentrics and translocations) abnormalities, whereas the numerical CAs consisted of polyploid cells. For the determination of the genotoxicity, only the structural CAs were taken into consideration.

The scoring of SCE was carried out according to the IPCS guidelines (Albertini et al. 2000). To score SCE, a total of 100 division metaphases per concentration (25 cells per donor) were analyzed. The results were used to determine the mean number of SCE (SCE/cell). In addition, a total of 400 cells (100 cells per donor) were scored to obtain the proliferation index (PI) for each treatment concentration. The PI was calculated according to $PI = [(M1 \times 1) + (M2 \times 2) + (M3 \times 3)]/\text{total scored cells}$. M1, M2, and M3 are the fraction of cells undergoing the first, second, and third mitosis during 72-h cell culture period. MI was determined by scoring 3,000 cells from each donor for each concentration.

In vitro cytokinesis-block micronucleus assay

For the analysis of MN in binucleated lymphocytes, 0.2 mL of fresh whole blood (1/10 heparinized) was used to establish the cultures and the cultures were incubated for 68 h. The cells were treated with 25, 50, 75, and 100 μg/mL concentrations of PMX for 24- and 48-h treatment periods. To block cytokinesis, cytochalasin B (Sigma, C6762) was added at 44 h of the incubation at a final concentration of 6 μg/mL. After an additional 24-h incubation at 37 °C, cells were initially harvested by centrifugation at 2,000 rpm for 5 min and further processed identically as described for the preparation of CA and SCE slides, with the exception of a 5 min hypotonic treatment step at 37 °C. Finally, the slides were stained with 5 % Giemsa (Kirsch-Volders et al. 2003; Rothfuss et al. 2000). In all subjects, 1,000 binucleated lymphocytes were scored from each donor (4,000 binucleated cells were scored per concentration). A total of 1,000 cells (4,000 cells for each treatment concentration) were scored to calculate the nuclear division index (NDI) for the cytotoxicity of PMX using the formula: $NDI = [(1 \times M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N$, where M1-M4 represent the number of cells with one to four nuclei and N is the total number of the cells scored (Fenech 2000).

Statistics

Values of the control, positive control and the exposed groups were expressed as the mean (±SE) from four separate experiments. The multiple comparisons between the control, the positive control and the exposed groups were performed using One-Way ANOVA (LSD test) at $p \leq 0.05$.

Results

The effects of PMX on CA formation in human peripheral blood lymphocytes are summarized in Table 1. In 24-h treatment, PMX significantly

Table 1 Effect of pemetrexed (PMX) on CA in human peripheral lymphocytes for 24- and 48-h

Test substance	Treatment		Structural CA		Polyploid cells	Percentage of cells with aberrations ± SE
	Time (hr)	Conc. (µg/mL)	Chromatid type	Chromosome type		
Control	–	–	3	5	1	2.00 ± 0.70
MMC	24	0.25	26	33	1	12.75 ± 1.43
PMX	24	25	7	15	1	5.25 ± 0.25 a_1b_3
PMX	24	50	7	11	0	5.00 ± 1.08 a_1b_3
PMX	24	75	8	18	0	6.25 ± 0.62 a_2b_3
PMX	24	100	5	16	0	5.00 ± 1.08 a_1b_3
MMC	48	0.25	199	61	1	42.75 ± 9.51
PMX	48	25	6	6	0	2.25 ± 1.03 b_3
PMX	48	50	3	10	0	3.00 ± 0.70 b_3
PMX	48	75	2	7	0	2.50 ± 0.64 b_3
PMX	48	100	1	3	0	1.00 ± 0.40 b_3

Data are expressed as the mean ± SE (n = 4)

a Statistically significant decrease vs. control, *b* Statistically significant vs. positive control

a_1b_1: $p < 0.05$; a_2b_2: $p < 0.01$; a_3b_3: $p < 0.001$

($p < 0.05$–0.01) increased the percentage of cells with aberrations at all concentrations in comparison with the control. However, the increase of the CAs at 24-h treatment period were not concentration-dependent. Noticeably, at 24-h treatment, the total number of chromosome type breaks for each concentration was approximately two-fold higher (three-fold difference at 100 µg/mL) as compared to the number of chromatid type breaks. In contrast, treatment with PMX for 48 h did not significantly induce the formation of CA when compared with the control.

24- and 48-h treatment with PMX did not induce a statistically significant SCE formation when compared to the control group (Table 2). However, some cells of the treated cultures at 25 µg/mL (24- and 48-h treatment) and 50 µg/mL (24-h treatment) gave an unexpected positive SCE response. The positive control MMC significantly induced SCE in comparison with all concentrations of PMX (Table 2).

Increasing PMX concentrations did not produce a significant increase in the percentage of binuclear cells with micronuclei or %MN for the 24- and 48-h treatment periods (Table 3). In addition, the %MNBN and %MN were significantly reduced at 100 µg/mL when compared with 25 µg/mL (Table 3).

PMX treatment for 24 h and 48 h significantly ($p < 0.01$–0.001) decreased the PI and MI in human peripheral blood lymphocytes (Table 4). Furthermore, PMX decreased the PI and MI to the same extent as the positive control MMC for both 24- and 48-h treatment periods. However, the observed reduction was not concentration-dependent. Furthermore, the majority of cells was undergoing the first-mitosis in the cultures treated with PMX for 48-h.

Table 2 Mean SCE values in human peripheral lymphocytes treated with PMX for 24- and 48-h

Test substance	Treatment		Min–Max SCE	SCE/cell ± SE
	Time (hr)	Conc. (µg/mL)		
Control	–	–	0–12	3.79 ± 0.35
MMC	24	0.25	0–67	36.27 ± 4.14
PMX	24	25	0–56	9.52 ± 3.09 b_3
PMX	24	50	0–43	4.73 ± 1.29 b_3
PMX	24	75	0–9	3.67 ± 0.48 b_3
PMX	24	100	0–12	3.42 ± 0.40 b_3
MMC	48	0.25	1–140	53.09 ± 6.41
PMX	48	25	0–75	7.14 ± 2.11 b_3
PMX	48	50	0–10	2.87 ± 0.20 b_3
PMX	48	75	0–10	3.32 ± 0.29 b_3
PMX	48	100	0–10	3.05 ± 0.33 b_3

Data are expressed as the mean ± SE (n = 4)

a Statistically significant vs. control, *b* Statistically significant vs. positive control

a_1b_1: $p < 0.05$; a_2b_2: $p < 0.01$; a_3b_3: $p < 0.001$

Table 3 Percentage of micronucleus (MN) and percentage of micronucleated binuclear (MNBN) cells in cultured human peripheral lymphocytes treated with PMX for 24- and 48-h

Test substance	Treatment		MN ± SE (%)	MNBN ± SE (%)
	Time (hr)	Concentration (μg/mL)		
Control	–	–	0.27 ± 0.09	0.25 ± 0.08
MMC	24	0.25	2.15 ± 0.25	2.02 ± 0.21
PMX	24	25	0.60 ± 0.07 b_3	0.60 ± 0.07 b_3
PMX	24	50	0.45 ± 0.17 b_3	0.42 ± 0.14 b_3
PMX	24	75	0.52 ± 0.13 b_3	0.50 ± 0.12 b_3
PMX	24	100	0.15 ± 0.06* b_3	0.15 ± 0.06* b_3
MMC	48	0.25	10.32 ± 2.72	10.32 ± 2.72
PMX	48	25	0.55 ± 0.11 b_3	0.55 ± 0.11 b_3
PMX	48	50	0.27 ± 0.06 b_3	0.25 ± 0.06 b_3
PMX	48	75	0.05 ± 0.03 b_3	0.05 ± 0.03 b_3
PMX	48	100	0.12 ± 0.06 b_3	0.10 ± 0.04 b_3

Data are expressed as the mean ± SE (n = 4)

* Statistically significant decrease vs.25 μg/mL for 24 h

a Statistically significant vs. control, *b* Statistically significant vs. positive control

a_1b_1: $p < 0.05$; a_2b_2: $p < 0.01$; a_3b_3: $p < 0.001$; * < 0.05

Finally, PMX caused a statistically significant ($p < 0.05$–0.001) reduction in the NDI for both 24- and 48-h treatment periods (Table 4). In addition, the decrease in the NDI after the 24-h PMX treatment was significantly ($p < 0.05$–0.01) greater than that caused by the positive control MMC.

Discussion

In the present study, we evaluated the genotoxic potential of PMX using CA and MN as the genetic endpoints, and the SCE test was used as an indicator of mutagenesis in human peripheral blood lymphocytes.

Table 4 Proliferation index (PI), mitotic index (MI) and nuclear division index (NDI) in human peripheral lymphocytes treated with PMX for 24- and 48-h

Test substance	Treatment		M1	M2	M3	PI ± SE	MI ± SE	NDI ± SE (%)
	Time (hr)	Conc. (μg/mL)						
Control	–	–	57	91	252	2.48 ± 0.78	7.43 ± 0.52	1.50 ± 0.13
MMC	24	0.25	99	228	73	1.93 ± 0.78	3.17 ± 0.15	1.34 ± 0.07
PMX	24	25	177	131	92	1.78 ± 0.15 a_2	3.29 ± 0.47 a_3	1.26 ± 0.05 a_1b_1
PMX	24	50	187	125	88	1.72 ± 0.14 a_3	3.53 ± 0.62 a_3	1.27 ± 0.06 a_1b_1
PMX	24	75	139	133	124	1.96 ± 0.09 a_2	3.25 ± 0.46 a_3	1.19 ± 0.02 a_2b_2
PMX	24	100	119	138	143	2.06 ± 0.05 a_2	3.73 ± 0.89 a_3	1.21 ± 0.04 a_1b_1
MMC	48	0.25	298	63	39	1.35 ± 0.09	1.59 ± 0.16	1.18 ± 0.22
PMX	48	25	146	158	96	1.87 ± 0.72 a_2 b_1	2.20 ± 0.49 a_3	1.06 ± 0.21 a_3
PMX	48	50	213	114	73	1.65 ± 0.16 a_3	2.81 ± 0.62 a_3	1.10 ± 0.26 a_3
PMX	48	75	204	112	84	1.70 ± 0.19 a_2	2.00 ± 0.51 a_3	1.11 ± 0.02 a_3
PMX	48	100	253	84	63	1.52 ± 0.16 a_3	1.61 ± 0.68 a_3	1.09 ± 0.24 a_3

Data are expressed as the mean ± SE (n = 4)

a Statistically significant vs. control, *b* Statistically significant vs. positive control

a_1b_1: $p < 0.05$; a_2b_2: $p < 0.01$; a_3b_3: $p < 0.001$

We also evaluated the effect of PMX on the cell division ratio, using MI, PI and NDI. Our results showed that PMX significantly increased the CA for 24-h treatment, but not for 48-h treatment. Interestingly, PMX increased neither %MN nor %MNBN in both 24- and 48-h treatment. PMX did not induce SCEs during both treatment periods. On the other hand, we observed a statistically very significant reduction in the MI, PI and NDI in PMX-treated peripheral blood lymphocytes in 24- and 48-h treatment periods.

We obtained a completely different cytogenetic toxicity profile with PMX compared to the previous reports on MTX that have shown a significant increase in CA both in the short and long term genotoxicity assays in vitro and in vivo. Chow et al. (1998) reported that CAs significantly increased in the NIH 3T3 mouse cell line after treatment with low doses of MTX. Similarly, the formation of CA and MN was found to increase in mice both in acute and chronic treatments with MTX (Choudhury et al. 2000, 2001; Kasahara et al. 1992a, b). Keshava et al. (1997) found a significant increase of CA in V79 cells treated with 5, 10, 25, 50 and 100 μg/mL of MTX after 6-h treatment. In that study, MTX increased the CA while decreasing the MI. However, in our study, we observed that PMX treatment caused a gradual decrease in the MI, which prevented the CA formation from reaching the statistical significance at 48-h treatment.

Hittelman (1973) demonstrated that 10^{-7} M of APT increased the chromatid type breaks in cultured potorous cells only at the first metaphase following treatment. Our results are in agreement with the results reported by Hittelman. Furthermore, we observed more chromosome type breaks as compared to the chromatid type breaks in peripheral blood lymphocytes treated with PMX for 24-h treatment. It was reported that the depletion of dTTP pools led to the misincorporation of uracil into the newly synthesized DNA, which is associated with the formation of chromosome breakage (Blount et al. 1997; Tonkinson et al. 1997). Our results are in agreement with those obtained earlier. However, the potent inhibition of multiple enzymes in the de novo biosynthetic pathway of thymidine led to a potent decrease of the MI, PI and NDI in 48-h treatment period, a mechanism which is responsible for the prevention of cells to enter mitosis bearing highly damaged DNA.

In this study, the exposure of human peripheral blood lymphocytes to the various concentrations (25, 50, 75 and 100 μg/mL) of PMX did not induce SCE either in 24- or 48-h treatment periods. However, an obviously greater SCE induction was observed in some cells of PMX-treated cultures (Table 2). It has been previously suggested that SCE induction at the single-cell level is a reflection of the differential sensitivity of lymphocyte subpopulations against DNA damage and SCE formation (Bender and Brewen 1969; Lezana et al. 1977). This suggestion had been further confirmed by Kelsey et al. (1988) in which they reported the existence of some lymphocytes cells with a high frequency with regard to SCE induction following ethylene oxide treatment in vivo. Thus, our findings confirm and extend earlier observations on the existence of drug-sensitive lymphocyte subpopulations and show that PMX is able to induce SCE. However, the persistence of cells bearing elevated SCE levels was decreased due to the death of cells with excessive chromosome damage.

Enzyme inhibition studies (Shih et al. 1998) showed that PMX exerts stronger inhibition of TS, DHFR and GARFT when compared to MTX, which inhibits primarily DHFR. Thus, in our study, statistically significant decline of the MI, PI and NDI in both 24- and 48-h treatment times is most probably associated with a strong inhibition of TS, DHFR and GARFT. In contrast, other studies did not report a significant decline in MI in MTX treated cells in vivo. Thus, we suggest that PMX has a novel and potent mechanism of cytotoxicity in comparison with MTX. It has been previously argued that MTX and its polyglutamated derivatives confer selective toxicity on tumor cells, due to an increased polyglutamate synthesis in tumor cells compared to normal proliferative cells (Poser et al. 1981). However, our results are not in agreement with this argument. PMX significantly decreased the mitotic division in normal human peripheral blood lymphocytes as evidenced by the strong decreases in MI, PI and NDI.

Data from unpublished studies (Tweats et al. 2007) showed that PMX significantly increased the number of micronucleated polychromatic erythrocytes in mouse bone marrow cells at the highest dose in 24-h treatment period. In contrast, we did not observe such a significant increase in either the %MN or the %MNBN for both 24- and 48-h treatment periods. Furthermore, %MN and %MNBN were significantly

decreased at the highest concentration (100 μg/mL) in the 24-h treatment period. The finding presented here strongly suggests that continuing cell death is responsible for the decrease in the DNA-damaging capacity of PMX. The accumulation of DNA strand breaks have been shown to be a cell-lethal event in MTX treated Ehrlich ascites tumor cells and was associated with a significant cytotoxicity after 10-h treatment (Li and Kaminskas 1984). Our results are in strong agreement with those obtained from the study of Li and Kaminskas. In contrast, the induction of CA without a concomitant decrease of the MI in mice bone marrow cells has been reported previously (Choudhury et al. 2000, 2001). The discrepancy between the in vitro and in vivo findings may be explained by the drug-induced deficiency in the DNA repair activity, which can not be tolerated in vitro.

The results of this study show that PMX causes damage in chromosomes of cultured human peripheral lymphocytes and rapidly decreases the MI, PI and NDI by killing cytogenetically damaged cells. Thus, we propose on the basis of its genotoxicity, that PMX treatment can be a safer medication in cancer treatment in comparison with other antifolates.

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