In vitro genotoxic perspective of Tamiflu

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Abstract The aim of this study was to investigate the genotoxic and/or cytotoxic effects of Tamiflu, commercial form of the oseltamivir antiviral and most frequently prescribed for the treatment of influenza infections, on cultured human peripheral lymphocytes by using sister chromatid exchange (SCE), chromosomal aberration (CA), and cytokinesis-blocked micronucleus (CBMN) assays. Cells were treated with 0.5, 1, 2 µg/mL oseltamivir, the Tamiflu capsule ingredient, for 24 or 48 h in the absence or presence of an exogenous metabolic activation system (S9 mix). The test chemical did not demonstrate any genotoxic effect dose-dependently but it showed a weak cytotoxicity on cells in this study. On the other hand, some concentrations of Tamiflu (2 µg/mL without S9 mix for 48 h and 1 µg/mL with S9 mix) induced SCE and also decreased significantly the proliferation index (PI) (48 h period) and the nuclear division index (NDI) (24 h period) ($P < 0.05$) in the absence of S9 mix. Considering the results, Tamiflu did not induce significant increases of CA or micronucleated cells in vitro in cultured peripheral blood lymphocytes under the treatment conditions used but weak SCE induction was observed. On the other hand, the weak cytotoxic effects observed disappeared in the cultures treated in presence of the S9 mix.

Keywords Tamiflu · Oseltamivir · In vitro human lymphocytes · Sister chromatid exchange · Micronuclei · Cytotoxicity

Introduction

Four major influenza pandemics had emerged in the twentieth century; Spanish influenza (1918–1919 influenza A H1N1), Asian influenza (1957–1959 influenza A H2N2), Hong Kong influenza (1968–1970 influenza A H3N2), and Russian influenza (1977–1978 influenza A H1N1). Spanish influenza was the most devastating one in the year 1918. It killed 20 million persons worldwide. In 1977, Russian influenza had a relatively minor effect and did not increase mortality (Ustaçelebi et al. 2004). Influenza is a serious illness that also causes labor force losses. Various solutions (like vaccine or antivirals) were suggested to cope with influenza. A group of them neuraminidase inhibitors is a new class of antiviral drugs that inhibit influenza A and B viruses (Winqvist et al. 1999). One of the most popular antiviral agents is oseltamivir, having a brand name of Tamiflu, within anti-influenza antivirals. Licensor of Tamiflu is Gilead Sciences, Inc. Foster City, California but it is distributed by Roche Pharmaceuticals (FDA 2008). Tamiflu is a member of neuraminidase inhibitor group drug; and it was approved in the year 1999. It was indicated that Tamiflu could be used confidently for everybody older than one year according to the leaflet of

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the drug, on the other hand, reliable and impartial data are unavailable in the related scientific literature concerning the genotoxicity of Tamiflu. Therefore, this study was performed to determine the effect of Tamiflu on cultured human peripheral blood lymphocytes by using worldwide well recognized genotoxicity assays.

Materials and methods

Agents

Tamiflu

The test substance—the Tamiflu drug—was obtained from the commercial pharmacy (CAS Registry Number: 196618-13-0). Synonyms are oseltamivir, tamvir, oseltamivir phosphate. One Tamiflu capsule, containing 75 mg oseltamivir that is the active ingredient was used as the test substance in this study. The chemical name is ethyl (3R,4R,5S)-4-acetamido-5-amino-3-pentan-3-yloxycyclohexene-1-carboxylate (molecular weight: 312.404520 [g/Mol]). The chemical structure of Tamiflu is as follows:

S9 preparation

To increase metabolizing liver microsomal enzymes activity (Gelboin et al. 1967), a male albino rat, 12 weeks old, weighing 200 g, was treated with 3-methylcholanthrene (80 mg/kg body weight; Oek-anal, Cat. no: 45794) by intraperitoneal route (Ueng et al. 2002). After, the treated rat was fed at light/dark periods for five days, then sacrificed and the liver was removed for preparing liver homogenates (the S9 fraction). The S9 fraction and the S9 mix thereof were prepared following the procedure described by Maron and Ames (1983). The S9 mix was prepared by a mixture S9 fraction and S9 pill. S9 pills containing NADP-Na₂, glucose-6-phosphate, Mg-aspartate, phosphate buffer pH 7.4, and KCl were purchased

from Roche (Cat. no: 1.745.425). Freshly prepared S9 fraction was divided into 1 mL portions in small plastic tubes; which were frozen immediately and stored at −80 °C. The S9 mix was prepared fresh for each genotoxicity assay; each S9 pill was dissolved in 18 mL of double-distilled water and this solution was supplemented with 2 mL of S9 fraction. Thus, the S9 mix was ready for use.

Assays

In vitro SCE and CA assays

The techniques by Evans (1984) and Perry and Thompson (1984) were applied with minor modifications for the preparation of chromosomes. This study was also organized in agreement with the IPCS (International Programme on Chemical Safety) guidelines (Albertini et al. 2000). The blood samples (0.2 mL) from two healthy donors (one female and one male, non-smokers, aged 24 years) were added to 2.5 mL chromosome medium B (Biochrom, F5023) for the *in vitro* CA test and supplemented with 10 µg/mL bromodeoxyuridine (Sigma, B5002) for the *in vitro* SCE test. These cultures were incubated at 37 °C for 72 h. The cells were treated during 24 or 48 h with three concentrations of Tamiflu, equivalent to 0.5, 1, or 2 µg/mL of oseltamivir antiviral agent. The concentrations were prepared using one Tamiflu capsule ingredient, containing 75 mg of oseltamivir. It was dissolved in DMSO (Sigma, CAS No: 67-68-5). The concentrations of the test compound used were 1/4 of the therapeutic dose, half of therapeutic dose, or the therapeutic dose. A solvent control (DMSO) and two positive controls, mitomycin-C (MMC, Kyowa, Hakko, Japan) and Cyclophosphamide monohydrate (Cyp, Sigma CAS No: 6055-19-2) were used. Volume of 0.5 mL S9 mix containing 50 µL of S9 fraction per culture tube was used for the cultures in which the metabolic activation was applied to detect indirect genotoxicity. Colchicine (0.06 µg/mL, Sigma C9754) was present for the last 2 h of the cell culture in order to arrest mitosis. To collect the cells, the cultures were centrifuged (306×g, 15 min), treated with a hypotonic solution (0.4% KCl) for 15 min at 37 °C, and then fixed in cold glacial acetic acid/methanol (1/3) for 20 min at room temperature. The treatment with the fixative was repeated three times with intermittent

centrifugation. The cells were then spread on cold glass slides and air-dried. The slides were stained with 5% Giemsa in accordance with the fluorescence plus Giemsa technique (Speit and Haupter 1985). Different types of structural and numerical aberrations were investigated by the examination of metaphases of 100 well spread samples for each donor. The frequency of SCE was investigated by the examination of 25 cells, under the second metaphase, from each donor. These results were used to determine the mean number of SCE (SCE/cell). Furthermore, 100 cells from each donor, total of 200 cells, were scored for proliferation index (PI). The PI was calculated by the following formula: $PI = ((1 \times M1) + (2 \times M2) + (3 \times M3)) / \text{total scored cells}$; where M1, M2, and M3 are the fractions of cells undergoing the first, second, and third mitosis, respectively, during the 72-h period of cell culture. MI was also determined by scoring a total of 3,000 cells from each donor.

In vitro CBMN assay

The micronucleus test was performed as described by Rothfuss et al. (2000). Peripheral blood (0.2 mL) from two healthy donors was added to 2.5 mL chromosome medium B and incubated at 37 °C for 68 h. The cells were treated with 0.5, 1, or 2 µg/mL of Tamiflu for 24 and 48 h. A solvent control (DMSO) and positive controls (0.25 µg/mL MMC and 28 µg/mL cyp) were also used. In order to block cytokinesis, cytochalasin B (Sigma, C6762) was added to the cultures, at a final concentration of 6 µg/mL, 44 h after initiating the cultures. A volume of 0.5 mL S9 mix containing 50 µL of S9 fraction per culture tube was added for cultures supplemented with metabolic activator (S9). Cultures were harvested 24 h later. Then, the cells were treated with a hypotonic solution (0.4% KCl) at 37 °C for 10 min, fixed once with a fixative (glacial acetic acid/methanol/0.9% NaCl = 1/5/6) for 20 min, and then fixed twice with fixative (glacial acetic acid/methanol = 1/5) for 20 min. The MN slides were air-dried overnight and stained with 5% Giemsa. One thousand binucleated lymphocytes were scored from each donor (2000 binucleated cells per concentration) to determine the MN frequency. A total of 500 intact cells were scored to determine the frequency of cells, with 1, 2, 3, or 4 nuclei, and calculate the NDI (nuclear division index) for cytotoxicity of agents 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 intact cells scored (Fenech 2000).

Statistical significance

Chromatid and chromosome gaps were not evaluated as chromosome aberrations (Mace et al. 1978). The significances between the percentages of means of SCE, cells with CA, the percentages of binuclear cell with MN, PI, MI, and NDI in treated cultures, and their controls were evaluated by using the *t* test. Dose–response relationships were determined from the correlation and regression coefficients for the percentages of cells with mean SCE, CA, and the percentage of binuclear cell with MN, PI, MI, and NDI.

Results

The effects of three concentrations (0.5, 1, and 2 µg/mL) of the test chemical on cultured human peripheral lymphocytes were evaluated in the presence or absence of the metabolic activation system for the treatment periods established. No evidence concerning genotoxic effects of Tamiflu was found in this study. These results are explained in detail in the following sections.

In vitro SCE, CA and MN assays without metabolic activation

Table 1 shows that the increases of SCE frequency were concentration-related for treatments lasting 24 h and 48 h, but no statistical significance was observed for several of the concentrations evaluated. Although a general tendency of Tamiflu to induce SCE in a dose-response manner was registered the SCE frequency was only significantly increased at 2 µg/mL ($P < 0.05$) in cultures treated for 48 h without the S9 mix in comparison to the control values.

Similarities between CA and cytokinesis-blocked micronucleus (CBMN) induction were observed (Table 2). The test agent did not cause the CA formation and did not produce MN formation in comparison to the controls.

Table 1 Frequency of SCE in cultured human lymphocytes treated with Tamiflu without S9 mix

Test chemical	Treatment		Min.–max. SCE	SCE/cell $\pm$ SE
	Period (h)	Concentration (μ g/mL)		
Control	24	3.7 μ L/mL	1–8	3.46 $\pm$ 0.06
MMC	24	0.25	26–86	45.58 $\pm$ 4.38
Tamiflu	24	0.5	1–10	3.38 $\pm$ 0.18
Tamiflu	24	1	2–11	3.74 $\pm$ 0.06
Tamiflu	24	2	1–8	3.98 $\pm$ 0.54
Control	48	3.7 μ L/mL	1–9	3.17 $\pm$ 0.11
MMC	48	0.25	14–83	39.7 $\pm$ 10.9
Tamiflu	48	0.5	1–7	2.99 $\pm$ 0.09
Tamiflu	48	1	1–7	3.64 $\pm$ 0.04
Tamiflu	48	2	1–10	3.76 $\pm$ 0.04 ^a

A total of 50 cells were scored for the SCE assay

Compared with the control group (DMSO), the difference is statistically significant

^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$

In vitro SCE, CA and CBMN assays with metabolic activation (S9 mix)

There were slight concentration-dependent increases of the SCE frequency but all of these increases were in general not statistically significant. A significant SCE increase was found only at 1 μ g/mL for the Tamiflu

Table 3 Frequency of SCE in cultured human lymphocytes treated with Tamiflu in presence of S9 mix

Test chemical	Treatment		Min.–max. SCE	SCE/Cell $\pm$ SE
	Period (h)	Concentration (μ g/mL)		
Control + S9mix	3	3.7 μ L/mL	1–9	3.76 $\pm$ 0.44
Cyp + S9mix	3	28	6–57	17.40 $\pm$ 4.70
Tamiflu + S9mix	3	0.5	1–15	3.84 $\pm$ 0.36
Tamiflu + S9mix	3	1	1–10	4.54 $\pm$ 0.02 ^a
Tamiflu + S9mix	3	2	1–13	5.03 $\pm$ 0.73

A total of 50 cells were scored for the SCE assay

Compared with the control group (DMSO), the difference is statistically significant

^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$

treatments in the presence of S9 mix when compared to the control ($P < 0.05$). All concentrations of Tamiflu tests did not induce SCE to the same level as the positive control sample (Cyclophosphamide monohydrate) (Table 3).

Neither CA nor CBMN increased with statistical significance when comparing the frequencies obtained for Tamiflu treatments in the presence of S9 mix with

Table 2 Frequency of chromosome aberrations (CA) and cytokinesis-blocked micronucleus (CBMN) in cultured human lymphocytes treated with Tamiflu without S9 mix

Test chemical	Treatment		CA $\pm$ SE (%)	CBMN $\pm$ SE (‰)
	Period (h)	Concentration (μ g/mL)		
Control	24	3.7 μ L/mL	8.00 $\pm$ 1.00	6.5 $\pm$ 0.50
MMC	24	0.25	51.00 $\pm$ 2.00	37.5 $\pm$ 8.50
Tamiflu	24	0.5	6.50 $\pm$ 0.50	5.5 $\pm$ 0.50
Tamiflu	24	1	9.00 $\pm$ 1.00	6.5 $\pm$ 0.50
Tamiflu	24	2	6.50 $\pm$ 0.50	6.5 $\pm$ 0.50
Control	48	3.7 μ L/mL	7.50 $\pm$ 0.50	6.5 $\pm$ 0.50
MMC	48	0.25	26.50 $\pm$ 7.50	107.0 $\pm$ 10.0
Tamiflu	48	0.5	7.50 $\pm$ 0.50	7.5 $\pm$ 0.50
Tamiflu	48	1	6.50 $\pm$ 0.50	8.5 $\pm$ 0.50
Tamiflu	48	2	7.50 $\pm$ 0.50	7.5 $\pm$ 0.50

A total of 200 cells were scored for the CA assay and a total of 2000 cells were scored for the CBMN assay

Compared with the control group (DMSO), the difference is statistically significant

^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$

Table 4 Frequency of chromosome aberrations (CA) and cytokinesis-blocked micronucleus (CBMN) in cultured human lymphocytes treated with Tamiflu in presence of S9 mix

Test chemical	Treatment		CA $\pm$ SE (%)	CBMN $\pm$ SE (‰)
	Period (h)	Concentration (μ g/mL)		
Control + S9mix	3	3.7 μ L/mL	8.50 $\pm$ 0.50	6.5 $\pm$ 0.50
Cyp + S9mix	3	28	14.00 $\pm$ 1.00	15.0 $\pm$ 1.00
Tamiflu + S9mix	3	0.5	7.50 $\pm$ 0.50	6.5 $\pm$ 0.50
Tamiflu + S9mix	3	1	7.50 $\pm$ 0.50	7.5 $\pm$ 0.50
Tamiflu + S9mix	3	2	12.00 $\pm$ 1.00	9.0 $\pm$ 1.00

A total of 200 cells were scored for the CA assay and a total of 2000 cells were scored for the CBMN assay

Compared with the control group (DMSO), the difference is statistically significant

^a $P \leq 0.05$, ^b $P \leq 0.01$; ^c $P \leq 0.001$

Table 5 Cytotoxic effects of Tamiflu in cultured human lymphocytes treated for 24 and 48 h without S9 mix

Compared with the control group (DMSO), the difference is statistically significant	Control	24	3.7 μ L/mL	1.91 $\pm$ 0.03	2.05 $\pm$ 0.05	1.313 $\pm$ 0.039
	MMC	24	0.25	1.85 $\pm$ 0.26	1.31 $\pm$ 0.48	1.245 $\pm$ 0.075
	Tamiflu	24	0.5	2.27 $\pm$ 0.10	2.43 $\pm$ 0.13	1.359 $\pm$ 0.059
	Tamiflu	24	1	2.15 $\pm$ 0.05	2.28 $\pm$ 0.02	1.251 $\pm$ 0.001 ^b
	Tamiflu	24	2	1.87 $\pm$ 0.24	1.83 $\pm$ 0.10	1.227 $\pm$ 0.023
	Control	48	3.7 μ L/mL	2.13 $\pm$ 0.12	2.61 $\pm$ 0.08	1.359 $\pm$ 0.035
	MMC	48	0.25	1.53 $\pm$ 0.01	1.46 $\pm$ 0.06	1.165 $\pm$ 0.001
	Tamiflu	48	0.5	2.09 $\pm$ 0.09	2.58 $\pm$ 0.22	1.314 $\pm$ 0.044
	Tamiflu	48	1	1.95 $\pm$ 0.01 ^a	2.35 $\pm$ 0.05	1.417 $\pm$ 0.101
	Tamiflu	48	2	1.84 $\pm$ 0.14	2.26 $\pm$ 0.03	1.656 $\pm$ 0.042

^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$

respect to the values obtained in the control cultures (Table 4).

Cytotoxic effect

A weak cytotoxic effect of Tamiflu was mainly observed in cultures treated without S9 mix. A reduction in the parameters for the evaluation of cytotoxicity such as, PI, MI and NDI was observed when comparing Tamiflu treatment with respect to the control cultures. In general, the cytotoxic effects were time and concentration-related, except for NDI for the cultures treated for 48 h, and showed statistical significance at some of the concentrations evaluated (Table 5). On the contrary, in the cultures treated in the presence of S9 mix no cytotoxic effects were observed. In general, the values of the cytotoxicity parameters were higher than those observed for the controls,

except for the PI, and no statistical significance was observed for any of the concentrations used (Table 6).

Therefore, the data suggest that the metabolism could exert a detoxification effect on metabolites generated from Tamiflu lowering the cytotoxic potential under the experimental conditions of used in this study.

Discussion

Many of antiviral drugs such as nucleoside analogues are characterized by potential safety concerns (including teratogenic, embryotoxic, carcinogenic, genotoxic and antiproliferative activities). In this study, the genotoxic and cytotoxic effects of Tamiflu, an antiviral drug belonging to the neuraminidase inhibitor group, were evaluated. The drug is known as a good solution for therapy of influenza infection. The dosage of Tamiflu

Table 6 Cytotoxic effects of Tamiflu in cultured human lymphocytes treated for 3 h in presence of S9 mix

Test chemical	Treatment		PI $\pm$ SE	MI $\pm$ SE	NDI $\pm$ SE
	Period (h)	Concentration (μ g/mL)			
Control + S9mix	3	3.7 μ L/mL	1.69 $\pm$ 0.10	1.65 $\pm$ 0.05	1.183 $\pm$ 0.109
Cyp + S9mix	3	28	1.54 $\pm$ 0.13	1.35 $\pm$ 0.05	1.116 $\pm$ 0.046
Tamiflu + S9mix	3	0.5	1.83 $\pm$ 0.11	1.74 $\pm$ 0.21	1.227 $\pm$ 0.137
Tamiflu + S9mix	3	1	1.53 $\pm$ 0.04	1.46 $\pm$ 0.23	1.193 $\pm$ 0.039
Tamiflu + S9mix	3	2	1.66 $\pm$ 0.09	1.83 $\pm$ 0.03	1.223 $\pm$ 0.055

Compared with the control group (DMSO), the difference is statistically significant

^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$

recommended by the European Medicines Agency (EMA/285148/ 2009) is 2 to 3 mg per kg body weight during an influenza pandemic (including children under the age of one). Moreover, the Committee for Medicinal Products for Human Use (CHMP) indicated that the benefits of using this medicine in pregnant or nursing mothers in case of an Influenza A/H1N1 pandemic outweigh the risks of its use (EMA/285148/ 2009). Zanamivir, also belonging to the neuraminidase inhibitor group, has neither genotoxic nor reproductive type toxicities (Freund et al. 1999). Nevertheless, there are no extensive data on genotoxicity of Tamiflu or its active ingredient oseltamivir. In this study, Tamiflu weakly induced SCE at the highest concentration with/without added S9 mix in cultured human peripheral lymphocytes. For this reason, it could be assumed to be a SCE inducer. SCEs can be increased by several agents that attack DNA. Likewise, the observation of SCE notably indicates effects of chemical mutagens and carcinogens on eukaryotic chromosomes. The first study dealing with this issue was carried out by Latt (1974) on human lymphocyte chromosomes treated with the bifunctional alkylating agent MMC that cross-links DNA. Additionally, Perry and Evans (1975) found that all direct-acting mutagens led to dramatically increased frequencies of SCEs in Chinese hamster ovary cells treated with 14 known or suspected mutagens/carcinogens. DNA topoisomerases inhibitors (like m-amsacrine, camptothecin, etoposide and nalidixic acid) also induced sister-chromatid exchanges in cultured human lymphocytes (Ribas et al. 1996). In parallel to the above information, the tested concentrations of our test substance evaluated in the present study also increased SCE frequency in cultured human lymphocytes both with and without S9 mix.

Nevertheless, clastogenic or aneugenic effects of Tamiflu were not detected in the cultured human lymphocytes; neither in the presence nor the absence of S9 mix. Likewise, Tamiflu did not induce significant increases in CBMN or chromosomal aberrations in the cultured cells. Despite the weak induction of SCE, clastogenic and aneugenic effects have not been recorded. The negative genotoxic effects observed in this study are in concordance with the non-carcinogenic potential of Tamiflu and their in vivo metabolites in rodents in a 2-year carcinogenicity study. Furthermore, negative results were obtained for Tamiflu in the following genotoxicity assays, with Ames test, in the chromosome aberration assay using cultured human lymphocytes and in the in vivo micronucleus test in mice (FDA Approved Drug Label). DNA damage appears to cause cell cycle arrest (Ward et al. 2000). Similarly, Tamiflu decreased the proliferation index and nuclear division index at some concentrations thus inferring it as being weakly cytotoxic, though this effect disappeared in the presence of S9 mix applications. This finding is important for showing the inefficiency of Tamiflu metabolites on the cell cycle.

As a conclusion, Tamiflu was found nongenotoxic and weakly cytotoxic in this study. However, its potential to induce SCE should be taken into consideration. According to the present study our test substance has revealed a number of suspicious results. We think that it should be further tested by impartial laboratories using other genotoxicity tests.

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