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Fosfomycin increases chromosome instability in lymphocytes from Fanconi Anemia patients

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ABSTRACT

Fanconi Anemia (FA) is a chromosome instability (CI) syndrome, clinically characterized by progressive bone marrow failure and increased cancer predisposition. Lymphocytes from FA patients have hypersensitivity to alkylating agents, particularly to diepoxybutane (DEB). The antibiotic fosfomycin (FOM) is an alkylating agent. FOM is used as a large spectrum antibiotic and also as a prophylactic pre-surgery agent. FOM has been considered non-genotoxic. However, no specific genotoxic evaluation directed to patients with hypersensitivity to alkylating agents was performed. As FA patients are very susceptible to infections and may be submitted to several surgeries, FOM can eventually be prescribed to them during their lifetime.

In the present study we evaluated the putative genotoxic effect of FOM in cultured lymphocytes from FA patients, compared to cultured lymphocytes from healthy donors (HD).

Cultures from FA patients and HD were treated with 0.5 mM FOM or with 0.6 mM DEB and CI was evaluated.

Results showed that FOM significantly increases CI in cultured lymphocytes from FA patients, compared to lymphocytes from HD, in which no effect was found. Additionally, a direct correlation between DEB and FOM toxicity was observed in lymphocytes from FA patients, indicating similar susceptibility to both agents.

1. Introduction

Fanconi Anemia (FA) is a rare genetic disease, characterized by chromosome instability (CI), and clinically featured by several congenital malformations, progressive bone marrow failure and increased cancer predisposition. Genetically, FA is a very heterogeneous disease; until now, several mutations have been identified in 15 genes (*FANC-A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P*) with functions mostly attributed to repair of DNA interstrand crosslinks (ICL) [1]. The cellular phenotype of FA is characterized by a unique hypersensitivity to DNA cross-linking agents, particularly to diepoxybutane (DEB) (Fig. 1), and this unique feature is routinely used for the cytogenetic diagnosis of FA [2].

It has been postulated that FA cells have a high sensitivity to oxidative stress (OS) [3–5]. Knowing that DEB toxicity is redox-related and oxygen dependent [6–8], the hypersensitivity of FA cells to DEB points to an important function of FA genes in the defense/repair against OS. In fact, the FA proteins FANC-A, C, D2, G and J were already associated with activities involved in detoxifying reactive oxygen species (ROS). Accordingly, the functions of FA proteins are currently associated to ICL repair, in response to the induction of OS-related DNA damage caused by exogenous agents, mainly alkylating agents, as reviewed in Ref. [9]. Therefore, environmental exposure of FA cells to alkylating agents can be a predisposition factor for an increased CI.

Fosfomycin (FOM) is a phosphonic acid derivate (cis-1.2-epoxypropyl-phosphonic acid) (Fig. 1). Like DEB, FOM is an aliphatic

Abbreviations: ATP, adenosine-5′-triphosphate; CI, chromosome instability; DEB, (±)-1,2:3,4-diepoxybutane; FA, Fanconi Anemia; FOM, fosfomycin; GSH, reduced glutathione; HD, healthy donors; ICL, interstrand crosslinks; IFAR, Fanconi Anemia Registry; MurA, enolpyruvyl transferase; OS, oxidative stress; PEP, phosphoenolpyruvate; ROS, reactive oxygen species; SmPC, summary of product characteristics; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine.

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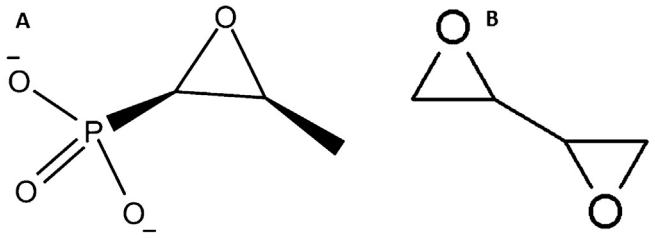

Fig. 1. Structures of fosfomycin (FOM) and (±)-1,2:3,4-diepoxybutane (DEB). Chemical struture of FOM (A) and DEB (B).

epoxide and, as most epoxides, it is an alkylating agent [10]. FOM is widely used as a broad-spectrum antibiotic, acting against a large variety of bacteria (Gram-positive and Gram-negative) for the treatment of several infections and as a prophylactic pre surgery agent. FOM has been considered as a well-tolerated antibiotic with a low incidence of adverse events [10–12].

It is known that some FA patients have an increase propensity to infections, due to the neutropenia associated with bone marrow failure. Additionally, FA patients are also submitted to several surgeries during their lifetime, mostly attributed to congenital malformations and to the high incidence of squamous cell carcinomas. It is therefore possible that these patients may be often exposed to antibiotics. A relevant information described in the Summary of Product Characteristics (SmPC) of FOM containing medicines is that this antibiotic is not considered mutagenic or genotoxic. However, there are no studies performed in patients hypersensitive to alkylating agents and therefore putative deleterious effects of FOM in these patients needs to be evaluated. In the present work the effect of FOM on the CI in cultured lymphocytes from healthy donors (HD) and FA patients was studied. A comparative study was also performed between FOM and DEB induced toxicity in lymphocytes from FA patients.

2. Methods

2.1. Patients and controls

Following informed consent, peripheral blood samples were collected from 12 healthy donors (HD), 5 males and 7 females (ages between 26 and 61 years), and 12 FA patients, 6 males and 6 females (ages between 1 and 37 years) that had been diagnosed with certainty on the basis of clinical features and cytogenetic findings. The spontaneous and FOM-mediated CI was studied in lymphocyte cultures from FA patients and HD. DEB-induced CI in lymphocyte cultures was evaluated only for FA patients.

2.2. Cells and cell cultures

Samples of 0.5 ml of whole blood were used for lymphocyte cultures in RPMI complete medium supplemented with 15% FCS and antibiotics. The cultures were stimulated with 5 μg/ml of phytohaemagglutinin (PHA, GIBCO, Invitrogen Corporation, USA) and placed in an incubator at 37 °C with a 5% CO2 atmosphere for 72 h.

After 24 h of culture, DEB ((±)-1,2:3,4-diepoxybutane, [298-18-0], D-7019 Lot 34H3683, Sigma Chemicals CO.) and FOM (disodium salt P5396 Lot BCBC4493, Sigma Chemicals CO), both prepared in RPMI, were added to appropriate lymphocyte cultures. DEB was added at a final concentration of 0.6 mM; since DEB is a suspected carcinogen with unknown risk, appropriate precautions were taken: all disposable culture bottles and pipettes were rinsed with HCl before being discarded. FOM disodium salt was added only at the concentration of 0.5 mM [12,19] to FA lymphocyte cultures (due to limitations in the volume of blood collected from each FA patient). Three concentrations of FOM were tested in HD cultures: 0.25, 0.5 and 1 mM.

2.3. Cytogenetic analysis

Following 3 days of culture, cells were harvested after 1 h incubation with colcemid (10 μg/ml), followed by hypotonic treatment with 75 mM KCl and fixation in a 1:3 solution of acetic acid: methanol. Chromosome preparations were made by the air drying method.

For each independent experiment, analysis of chromosome aberrations was performed by one scorer on 50 Giemsa-stained metaphases from coded slides (only for one patient 24 metaphases were scored). To avoid bias in cell selection, consecutive metaphases that appeared intact, with sufficient well-defined chromosome morphology, were selected for study. Each cell was scored for chromosome number and the number and types of structural abnormalities. Achromatic areas less than a chromatid in width were scored as gaps while those wider than a chromatid were scored as breaks. Chromatid exchange configurations (like triradial and tetraradial figures), dicentric and ring chromosomes were scored as rearrangements. Gaps were excluded in the calculation of chromosome breakage frequencies and rearrangements were scored as two breaks. Chromosome breakage parameters (% aberrant cells and no. of breaks/cell) were determined according to the International Fanconi Anemia Registry (IFAR) protocol [20].

Table 1
Fosfomycin-induced chromosome instability in cultured lymphocytes from 12 healthy donors.

Group	% Aberrant cells		Nr breaks/cell	
	Mean	Standard deviation	Mean	Standard deviation
Control	4.83	2.89	0.05	0.03
Fosfomycin 0.25 mM	3.83	3.24	0.04	0.03
Fosfomycin 0.5 mM	3.67	2.81	0.04	0.03
Fosfomycin 1 mM	4	2.95	0.04	0.03

FOM – induced CI was evaluated in cultured lymphocytes from 12 HD healthy donors, measured by the percentage of aberrant cells and by the number of breaks per cell. Three concentrations of FOM were tested: 0.25, 0.5 and 1 mM.

2.4. Mitotic index

A comparative study of the mitotic index (MI) was measured in lymphocyte cultures from 7 patients and 7 healthy donors, with and without FOM, was performed. Microscope stained slides were prepared, a thousand cells were randomly scored, distinguishing between interphase nuclei and metaphase nuclei. MI was calculated as follows: number of metaphase nuclei/number of total nuclei. Once no significant differences were observed for the first 14 subjects studied, no MI was measured in lymphocyte cultures from the remained 5 FA patients and 5 HD.

2.5. Statistical analysis

Graphical results are expressed as mean ± SD and tabular results are expressed as mean ± SD. Statistical comparison among groups was estimated using one-way ANOVA, followed by the Bonferroni *post hoc* test and comparison between two groups was estimated using paired *t*-test. The correlations were performed by calculating the Pearson correlation coefficient, *r*, and a corresponding *P* value, using GraphPad Prism, version 5.0 software. *P* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Influence of fosfomycin on the frequency of chromosome instability in lymphocyte cultures from healthy donors

Exposure of cultured lymphocytes from HD to FOM did not elicit any genotoxic effect (Table 1 and Fig. 2). In fact, no significant differences were observed, for all concentrations tested (0.25, 0.5 and 1 mM), in the percentage of aberrant cells.

3.2. Influence of fosfomycin on the frequency of chromosome instability in lymphocyte cultures from FA patients

Contrasting the results obtained for HD, exposure of cultured lymphocytes from FA patients to FOM, at the concentration of 0.5 mM, elicited a significant increase in the number of breaks per cell and in the percentage of aberrant cells, when compared with the control group ($^{**}P < 0.05$ and $^{**}P < 0.05$ respectively) (Table 2).

3.3. Comparative study between fosfomycin and DEB induced chromosome instability

A comparative study was performed between the hypersensitivity of FA lymphocytes to the alkylating agents DEB and FOM. As shown in Table 3 and Fig. 2, a significant correlation was observed