Research Article

Genoprotective effects of Amifostine against Mitoxantrone induced toxicity in HepG2 cells, detected via the Comet assay

Mohammad Shokrzadeh, Nasrin Ghassemi-Barghi*

Faculty of pharmacy, Department of Pharmacology and Toxicology, Pharmaceutical Research Committee, Mazandaran University of Medical Sciences, Sari, Iran

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Abstract

Background: Mitoxantrone is involved in secondary malignancy due to its genotoxic potential. It can cause single and double strand breaks induction and inhibition of DNA synthesis, increase in reactive oxygen species (ROS) production and transient depletion of intracellular glutathione. Amifostine is a cytoprotective adjuvant used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents. It has been shown to exert an important cyto-protective effect in many tissues and directly limit cell injury and ROS generation during oxidative stress. Also, amifostine exerts anti-inflammatory and anti-apoptotic effects in several systems. **Objective**: The aim of this study was to explore whether amifostine protects against mitoxantrone-induced genotoxicity. **Materials and Methods:** For this purpose, cells were incubated with amifostine and mitoxantrone in pre and co-treatment condition and then then genoprotective effects of amifostine have been determined via comet assay. Oxidative stress was measured with intracellular reactive oxygen species and glutathione levels. **Results and conclusion:** Our results showed that mitoxantrone induced a noticeable genotoxic effects in HepG2 cells ($p<0.0001$). Amifostine reduced the effects of mitoxantrone significantly ($p<0.0001$) by reduction of the level of DNA damage via blocking ROS generation, and enhancement of intracellular glutathione levels.

***Keywords**:* Mitoxantrone, Amifostine, genotoxicity, comet assay, reactive oxygen species

Introduction

Mitoxantrone is a synthetic anthracenedione and anthracycline analog with antineoplastic effect (Thakur, 2011). Mitoxantrone, as an topoisomerase II (TOP2) inhibitor, is used in the treatment of various types of cancers such as metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma (Blasiak et al., 2002). It was also shown to increase the survival rate of children suffering from first relapse of acute lymphoblastic leukemia (Waters et al., 2010). Mitoxantrone in combination with prednisone is used as a second-line treatment for metastatic hormone-refractory prostate cancer. Decelerating the progression of secondary-progressive multiple sclerosis (MS), is another application of mitoxantrone (Scott and Figgitt, 2004). Side effects of treatment include myelosuppression, neutropenia, cardiomyopathy, nausea, vomiting and induction of secondary tumors (Blasiak et al., 2002). Moreover treatment with mitoxantrone can lead to renal dysfunction, cardiotoxicity and genotoxicity (Boos and Stopper 2000; Kluza al., 2004; Thakur, 2011). Binding of mitoxantrone to topoisomerase II results in cleavable complexes that generate DNA strand breaks, inhibits DNA replication and RNA transcription in a cell cycle nonspecific manner (Nitiss, 2009; Khan et al., 2010). Mitoxantrone cause apoptosis, mitochondrial dysfunction, and free radical generation in cardiomyocytes as well as tumorous cells (Kluza et al., 2004). The ability to induce DNA damage in normal cells and the induction of secondary malignancies may be considered as the most critical side effects of anticancer drugs (Blasiak et al., 2002, Chronowski et al., 2004). The genotoxic effects of mitoxantrone have been

Address for Corresponding Author:
Nasrin Ghassemi Barghi
Department of Toxicology and Pharmacology,
Faculty of Pharmacy Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran
E-mail: ngeternal67@yahoo.com; mslamuki@yahoo.com

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proven by chromosomal aberration tests, micronucleus assay and Comet assay in various studies (Suzuki and Nakane 1994; Boos and Stopper, 2000; Blasiak et al., 2002). Thus, a thorough assessment aimed to its side effects, like genotoxicity which leads to secondary malignancy is required.

Amifostine, is a cytoprotective agent used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents (Kanat et al., 2003). Amifostine is an inactive prodrug that cannot protect cells until dephosphorylated to the active metabolite, WR-1065, by alkaline phosphatase in the plasma (Stankiewicz et al., 2002). According to the different studies, inside the cell, amifostine's protective effects appear to be mediated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia, the release of endogenous nonprotein sulfhydryl's (mainly glutathione) from their bond with cell proteins and formation of mixed disulphides to protect normal cells (Torres and Simic, 2012). Amifostine has shown significant radio- and chemoprotective effects in several in vitro and in vivo studies. It is presently accepted for clinical use as a protective agent against renal toxicity induced by cisplatin in patients being treated for ovarian cancer and against xerostomia induced by ionizing radiation in patients with head and neck cancer (Santini and Giles 1999; Hartmann et al., 2000; Antonadou et al., 2002; Arany and Safirstein, 2003). Preclinical studies have shown that administration of amifostine before irradiation protected against radiation clastogenesis, mutagenesis and carcinogenesis (Sanderson and Morley, 1986; Damron et al., 2000). Amifostine is able to inactivate electrophilic substances and scavenge free radicals (Marzatico et al., 2000). In addition numerous studies has been showed that amifostine attenuate cardiotoxicity, nephrotoxicity and genotoxicity result from chemotherapy agents (Hartmann et al., 2000; Buschini et al., 2002; Gloc et al., 2002; Dragojevic-Simic et al., 2004).

Single cell gel electrophoresis (comet assay) is considered as a sensitive method for analyzing genotoxic or genoprotective potential of compounds is normally used in genotoxicity testing. Applications of this test include genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as primary research in DNA damage and repair (Hartley et al., 2011; Ghassemi-Barghi et al., 2016). The purpose of present study was to explore the protective effect amifostine against mitoxantrone induced genotoxicity. For this purpose we measured the DNA damage level with comet assay in HepG2 cells treated with mitoxantrone and amifostine in co and pre-treatment conditions. We also investigated the generation of ROS and intracellular glutathione levels as possible genotoxic mechanisms.

Materials and methods

Chemicals

Mitoxantrone was purchased from sigma-Aldrich, France. Amifostine, EDTA, H_2O_2, NaCl, NaOH, Na_2CO_3, NaH_2PO_4, Tris, and Triton X-100 were acquired from Merck Co. (Germany). Low melting point agarose (LMA), Na2HPO4, KCl and ethidium bromide were from Sigma Co. (USA). Normal melting point agarose (NMA) was supplied by Cinnagen Co (Germany). The RPMI 1640 medium, fetal bovine serum (FBS) and antibiotic was purchased from biosera (France). DCFH-DA probe and mBCl were from sigma Aldrich (USA) And, HepG2 cells came from Pasture Institute (Iran). All other chemicals used were of analytical grade.

Cell culture

Human hepatoma (HepG2) cells was obtained from Pasture Institute of Iran were grown as monolayer culture in RPMI 1640 medium supplemented with 10% FBS, 1% of mixture of penicillin (100 IU/ml) and streptomycin (100µg/ml) incubated at 37°C in an atmosphere of 5% CO_2–95% air. . The studied concentrations of mitoxantrone and amifostine were diluted in cell culture medium. We have chosen untreated cells as control. Cells were seeded on 24-well culture plates at 25×10^4 cells/well, after overnight growth, cells treated with studied concentrations of amifostine (1, 5 and 10 mg/ml)24 h prior to mitoxantrone treatment (0.05µM) for 1 h at 37°C (Etebari et al., 2012; Etebari et al., 2012).

Single-cell gel electrophoresis (SCGE, the comet assay)

Comet assay was performed under alkaline conditions according to the methods of Singh et al with slight modifications. HepG2 cells seeded at 25×10^4 cells/well. To examine the genotoxic effect, the cells were treated with mitoxantrone for 1h and to examine the anti-genotoxic effect, the cells were treated with amifostine in co and pretreatment conditions. Untreated cells considered as a negative control. Microscope slides (frosted) were covered by a thin layer of 1.0% normal-melting point agarose which. Upon solidification of the agarose, 10µL of a freshly prepared suspension of treated or control HepG2 cells were mixed with 100µL of low-melting-point agarose. The cells were covered with a coverslip and incubated at 4 °C for 10 min. After solidification of the agarose, the coverslips were removed and the slides were incubated in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10], 1% Triton X-100 and 10% DMSO) at 4°C for 40 minutes. After lysis, the slides were placed in a horizontal electrophoresis unit containing alkaline electrophoretic solution (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4°C with an

electric field strength of 25 V (0.78 V/cm) and a current of 300 mA, which allows the DNA to unwind, exposing alkali-labile sites. The slides were then washed with cold neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min. After drying at room temperature, the slides were stained with 30μL ethidium bromide (20μg/mL) and immediately analyzed using a fluorescence microscope equipped with a 515–560 nm excitation filter, a 590 nm barrier filter and an integrated digital camera. Slides were evaluated using Comet Score software (TriTek Corp., USA), and 100 randomly selected nucleoids (50 nucleoids from each replicate slide) were analyzed per treatment. Three independent experiments were performed. DNA damage was quantified by the percentage of DNA in the comet tail (% DNA)(Ghassemi-Barghi et al., 2016; Ghassemi-Barghi et al., 2017).

Measurement of Oxidative Stress

Approximately 4×10^4 cells per well were cultured for 24 h in 96-wellplates (black-wall/clear-bottom). Thereafter, the medium was aspirated, and the cells were washed twice with HBSS. The cells were then treated with studied concentrations of amifostine (1, 5 and 10 mg/ml) 24 h prior mitoxantrone treatment (0.05μM) for 1 h at 37°C. After the treatment, cells were washed twice with HBSS and incubated in 2 ml of fresh culture medium without FBS. 2, 7 Dichlorodihydrofluorescein diacetate was added at a final concentration of 10μM and incubated for 20 min. The cells were then washed twice with PBS and maintained in 1 ml of culture medium. Assess ROS by immediately analyzing cells by fluorescence plate reader using the 488 nm for excitation and detected at 535 nm. We have chosen untreated cells as a negative control and cells treated with 0.1 mM H_2O_2 as a positive control (Wang et al., 2004; Shokrzadeh and Ghassemi-Barghi, 2018).

Measurement of intracellular GSH levels

HepG2 cells were plated in a 96-well plate at 50,000 cells/well. After overnight growth, they were treated with amifostine for studied concentration (1 ,5 and 10 mg/ml) and busulfan (0.05μM) in pretreatment condition, then incubated with monochlorobimane (mBCI, 40 μM) in a staining solution (5mMglucose, 1 mM $CaCl_2$, 0.5mMMgSO_4, 5 mg/ml BSA) for 30 min at 37°C in the dark. Although mBCI is a nonfluorescent probe, it forms a stable fluorescent adduct with GSH in a reaction catalyzed by the GSH S-transferases .The mean fluorescent intensity of the fluorescent GSH-bimane adduct was measured using a Spectra fluorescent plate reader at λex=380 nm and λem=460 nm to detect GSH (Hedley and Chow, 1994).

Statistical analysis

Tail moment (percentage of DNA in tail × tail length), tail length (the length of the comet tail), and percent of DNA in tail (percentage of colored spots in tail) are the most frequently used factors in the evaluation of DNA damages in the comet assay method. We used these factors for statistical analysis in this investigation. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test was used to compare the results of the all assays.

Results

Study the effect of amifostine on mitoxantrone-induced DNA damage

The anti-genotoxic effect of amifostine was investigated through the alkaline comet assay. Results of the visual scoring and percentage of total DNA damage induced by mitoxantrone and prevented by amifostine were shown in Table 1. We observed that mitoxantrone treatment at 0.05μM induced a significant ($p < 0.001$) increase in DNA damage as compared to the control group. Amifostine in the different treatment conditions decreased significantly ($p < 0.0001$) the level of DNA fragmentation as compared to the mitoxantrone group.

Table 1. The genoprotective effect of Amifostine compared with control groups on tail length (pixels), percentage of DNA in tail, and tail moment (pixels) that are represented as mean± SEM. * and # mean value was significantly different from control and co-treatment group ($p < 0.0001$),(one-way ANOVA followed by tukeys post hoc test).

Treatment groups		Tail length (Mean±SEM)	%DNA in Tail (Mean±SEM)	Tail moment (Mean±SEM)
Pre-treatment	Control (mX0.05μM)	124.1±4.1	62.51±1.9	59.8±2.19
	Amifostine (1mg/ml)	75.83±3.8	32.3±1.2	42.3±1.6
	Amifostine (5mg/ml)	34.23±1.8 *	21.1±0.2*	2.4±1.1*
	Amifostine (10mg/ml)	17.4±1.13*#	10.3±0.5*#	0.2±.023*#
Co-treatment	Control (mX0.05μM)	124.1±4.1	62.51±1.9	59.8±2.19
	Amifostine (1mg/ml)	53.1±1.9	43.91±1.5	47.1±1.2
	Amifostine (5mg/ml)	41.1±2.1*	29±0.42*	4.1±1.11*
	Amifostine (10mg/ml)	23.19±1*	15.1±0.22 *	0.7±.01*

Study the effect of amifostine on ROS generation in mitoxantrone-treated cells

To investigate the role of oxidative stress in mitoxantrone-induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to mitoxantrone stimulation. Incubation with mitoxantrone for 1 h showed a considerable increase in oxidant-induced 2, 7-dichlorofluorescein fluorescence in HepG2 cells (Figure 1). H2O2-mediated DCF fluorescence occurred after 1h incubation with mitoxantrone (0.05µM) in HepG2 cells. This suggests that mitoxantrone, induce intracellular oxidative stress, involved in its genotoxicity. After that cells were treated with amifostine in pre-treatment condition and subsequently examined. Amifostine was significantly ($p<0.0001$) reduced ROS generation as compared to the busulfan group. Untreated cells served as control

Study the effect of mitoxantrone on intracellular levels of GSH

We first examined the effect of mitoxantrone on the intracellular levels of GSH using mBCI which readily enters cells to form a fluorescent GSH-bimane adduct that can be measured fluorometrically. As shown in figure 2, within 1h after mitoxantrone treatment, the intracellular levels of GSH were reduced ($p<0.0001$). This finding was subsequently confirmed by an enzymatic assay using glutathione reductase and 2-vinylpyridine. Next, we measured the intracellular levels of GSH in cells after treatment with amifostine and mitoxantrone in pre-treatment condition. As shown in figure 2 amifostine were significantly ($p<0.0001$) increased GSH levels as compared to the mitoxantrone group.

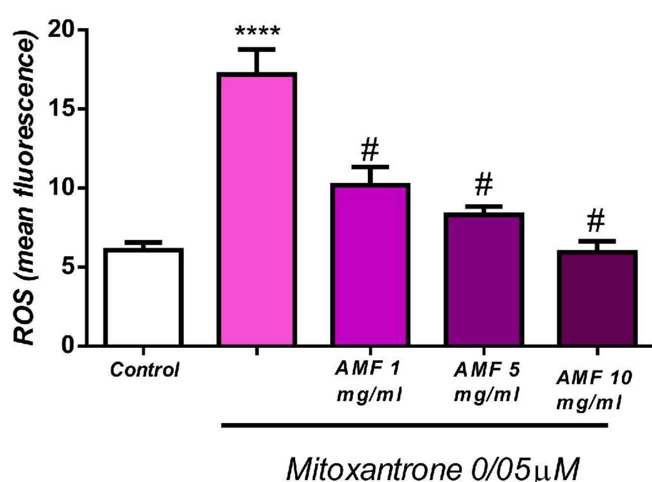

Figure 1. Study the effect of amifostine on mitoxantrone-induced ROS generation. (****) show significantly increased results (respectively $p<0.0001$) as compared to the control group. The sign (#) show significantly ($p<0.0001$) decreased compared to the mitoxantrone group.

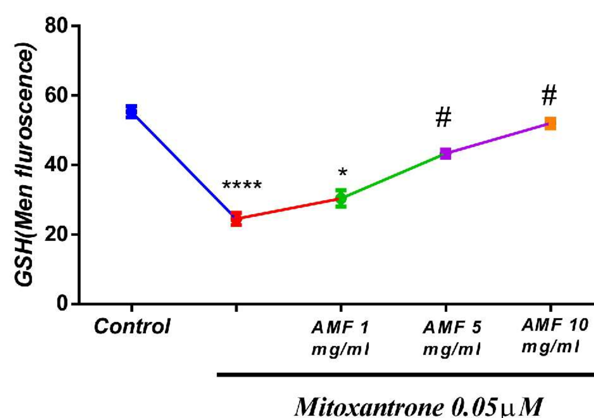

Figure 2. The effect of amifostine on the levels of intracellular GSH were determined. ANOVA analysis revealed that amifostine, significantly inhibited the effects of mitoxantrone on the levels of GSH. Sign (****) and (*) show significantly decreased results (respectively $p<0.0001$and $p<0.05$) as compared to the control group. Sign # show significantly ($p<0.0001$) increased as compared to the mitoxantrone group.

Discussion

Topoisomerase II contains a family of nuclear enzymes that are necessary for all living cells. Type II topoisomerases play an important role in DNA metabolic procedures, in which they are involved in DNA replication, transcription, chromosome condensation, DNA recombination, and untangling of replicated chromosomes. Topoisomerase II is the cellular target for a number of extensively used anticancer agents presently in clinical use such as mitoxantrone (Scott and Figgitt, 2004). It is mainly used in treatment of metastatic breast cancer, prostate cancer, lymphoma and subset secondary progressive multiple sclerosis (MS). As a topoisomerase II inhibitor, mitoxantrone disrupts DNA synthesis and DNA repair in both healthy and cancer cells by intercalation between the DNA bases. However, treatment with mitoxantrone shows noteworthy adverse effects such as cardiotoxicity, myelosuppression, leukopenia, kidney failure and extravasation, in dose-dependent manner (Bellosillo et al., 1998, Fox 2004). Mitoxantrone cause mitochondrial dysfunction and apoptosis in H9C2 cardiomyocytes cell line (Kluza et al., 2004). Other findings propose that mitoxantrone induces toxicity by hydroquinone oxidation, resulting in ROS generation. Therefore, mitoxantrone damages cell mitochondria, arrests cell cycle and conclusively cause apoptosis in normal cell lines. Inhibition of topoisomerase II by mitoxantrone can result in DNA damage and is a serious signal for NF-kappa B activation

and induction of apoptosis but the apoptosis does not increase DNA damage.

Amifostine, is the most effective radioprotector known and the only one approved for clinical use in cancer radiotherapy (Rades et al., 2004). This antigenotoxic effect was explained by assuming a high affinity of amifostine for DNA, thus stabilizing the DNA molecule and facilitating the activity of DNA repair enzymes (Majsterek et al., 2005). Preceding studies using mammal cells have shown that amifostine improves DNA repair and therefore improves cell survival. Amifostine phosphorylated aminothiol, also is an antioxidant clinically approved to prevent the neutropenia-associated events in patients receiving alkylating agents (Lorusso et al., 2003). In experimental animals a study showed that treatment with AMF effectively protects normal tissue from the toxicity of therapeutic radiation, without protecting tumorous cells (Ben-Josef et al., 2002). Nagy et al., subsequently showed that AMF showed the protective effect against the mutagenicity of cisplatin, assessed by the mutation rate of HPRT in V79 Chinese hamster cells (Camelo et al., 2008). Other reports recognized that amifostine protects normal tissue against radiation-induced damage by increasing intracellular SOD2 activity (Dziegielewski et al., 2008). Moreover, in another study found that WR1065, the active free thiol form of amifostine, induces antioxidative capacity against radiation via SOD2 *in vitro* (Khodarev et al., 2004). Other studies have been shown the role of SOD2 in amifostine-induced protective effects SOD2 mediated amifostine-induced antioxidative actions in PC12 cells exposed to glutamate. As SOD2 protein is mainly expressed in mitochondria which have been identified as a major source of ROS, we infer that high level of SOD2 protein may protect mitochondria by consuming ROS generated in oxidative injury. In addition, SOD2 mediated amifostine-induced effects on intracellular ROS, CAT, and GSH levels, indicating SOD2 may be the key target of amifostine in maintaining the balance of intracellular oxidants and antioxidants in PC12 cells. In our investigation we quantified the DNA-damage level, to clarify the possible anti-genotoxic mechanism of amifostine against mitoxantrone-induced toxicity in $HepG_2$ cell line. Our results showed that mitoxantrone caused a significant increase in DNA fragmentation as compared to the untreated cells. However, treatment of HepG2 cells with amifostine 24 h before mitoxantrone administration induced a noticeable decrease in DNA fragmentation as compared to the mitoxantrone X-treated group. Measurement of ROS generation showed that mitoxantrone induced ROS generation. Amifostine is a potent cytoprotective agent that can inhibit oxidative stress by scavenging ROS and replenishing GSH.

Conclusion

In conclusion, we have demonstrated that amifostine protected HepG2 cells against mitoxantrone-induced DNA damage and oxidative injury. Furthermore, we showed that mitoxantrone increased intracellular ROS generation and decreased intracellular GSH levels. Amifostine ameliorated the balance of intracellular antioxidants and oxidants, decreased ROS generation and enhanced the intracellular level of GSH.

Conflicts of interest

Authors declare no conflict of interest

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