Modulation of Mitomycin C-induced Genotoxicity by Acetyl- and Thio- Analogues of Salicylic Acid

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Abstract. Background: Recent reports regarding acetylsalicylic acid (ASA) and its metabolites suggest suppressive effects against mitomycin C (MMC)-induced genotoxicity in a mice chromosomal aberration assay. Keeping this in mind, the potential anti-genotoxic effect of the thio-analogue of salicylic acid namely thio-salicylic acid (TSA) was speculated upon. The present study investigated and compared the anti-genotoxic potential of ASA and TSA. Materials and Methods: The study was performed in male swiss mice (20±2 g) using single-cell gel electrophoresis and a peripheral blood micronucleus assay. ASA and TSA (5, 10 or 20 mg/kg) were administered 15 minutes after MMC (1 mg/kg) once daily for 3 or 7 days. Results: Both ASA and TSA significantly decreased the DNA damage induced by MMC as indicated by a decrease in the comet parameters in bone marrow cells and decreased frequencies of micronucleated reticulocytes in peripheral blood. Conclusion: The results clearly demonstrate the anti-genotoxic potential of ASA and TSA.

Aspirin, acetylsalicylic acid (ASA), is much explored as an anti-inflammatory, antipyretic and analgesic agent. Niikawa et al. revealed anti-genotoxic potential of ASA and its metabolites (1). It is reported to induce synthesis of the antioxidant defence protein, ferritin, which sequesters iron which otherwise catalyses damaging oxidative reactions (2). Nucleophilic sulphur compounds can be reduced to provide an alternative nucleophilic target and can thereby inactivate electrophilic intermediates. Unreduced nucleophilic sulphur compounds can also bind to other reactive species such as oxygen free radicals, which mediate damage to normal tissue following exposure to a variety of agents (3). Chemoprotection by thiols is presumed to be mediated by inactivation of the charged carbonium ions of activated alkylating agents through a nucleophilic attack, thereby protecting the nucleic acids from alkylation (4-6). Thio-salicylic acid (TSA) is a structural analogue of salicylic acid and belongs to a special class of thiols, where the carbonyl function is located two carbons away from a thiol group in a co-planar orientation. This induces TSA to act as an active thiol and a free thiol donor, like cysteine and N-acetyl-cysteine (7, 8). Such a thiol group provides sites for formation of co-ordinate bonds to metal ions and electrophilic reactions, as well as being an easily reversible site for redox reactions. Thus, it is speculated that being an active thiol, this structural analogue of salicylic acid can act as a better free radical scavenger and an alternative nucleophilic target for reactive electrophilic intermediates of mitomycin C (MMC). In the present study, an attempt has been made to investigate and compare the protective effects of ASA and TSA against MMC-induced DNA damage using comet and micronucleus assays as the end-points.

Materials and Methods

Animals. Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC). Animals were obtained from the Central Animal Facility of the Institute and pelleted diet and water were provided ad libitum. Male swiss albino mice (20±2 g) were used in the study and were housed five per cage under automatically controlled temperature (22±2°C), humidity (50±10%) and light (0600 to 1800 h) conditions.

Chemicals and dose administration. Mitomycin C (CAS No. 50-07-7, Batch No. 041201) was purchased from Cadila Pharmaceuticals (P) Ltd. Ahmedabad, India. Aspirin (CAS No. 50-78-2), thiosalicylic acid (CAS No. 147-93-3) and carboxymethylcellulose (CMC; CAS No. 9004-32-4) were purchased from Sigma-Aldrich, USA. Aqueous suspensions of ASA and TSA were freshly prepared for each treatment using CMC (0.5% w/v) and were administered.
through the oral route (p.o.). An aqueous solution of MMC freshly reconstituted for each treatment was administered through the intraperitoneal route (i.p.). MMC was administered once daily followed by a single dose of ASA/TSA administered 15 min after each dose of MMC over a period of 3 or 7 days. Mice administered 0.5% CMC served as vehicle-treated controls.

**Micronucleus test.** Genotoxic effects were evaluated in the mouse peripheral blood using the micronucleus test essentially as described with some modifications (9). The slides were stained with Acridine Orange (AO). Observations were made within 24 h after staining using an Olympus fluorescence microscope (Model BX 51) in association with digital photomicrograph software (OLYSPIA Imaging System, 2001). For each animal, the frequency of micronucleated reticulocytes (MNRETs) in peripheral blood smears was determined per 1,000 reticulocytes.

**Comet assay.** Bone marrow cells were isolated essentially as described with some modifications (10). From the bone marrow suspension (2-5×10⁴ cells/mL), 5 μL were mixed with 95 μL of 0.5% low melting agarose to prepare the final cell/agarose suspension. Base slides were coated with 1% normal melting agarose. A second layer of cell/agarose suspension (80 μL) was poured over the base slides. Immediately, the coverslips were placed on the agarose layer followed by a third layer of agarose (80 μL LMA) and these slides were placed in lysis buffer for 24 h. Subsequently, the slides were rinsed three times with distilled water and incubated in fresh alkaline buffer (pH>13) for 30 min. Electrophoresis of DNA was performed for 30 min at 300 mA and 25 V (0.90 V/cm). The slides were neutralized with 0.4 M Tris (pH 7.5) and then stained with ethidium bromide (2 μg/mL). Slides were scored using a Carl Zeiss (AXIO), Imager M1 microscope connected to a computer with Comet Imager V.2.0.0 software for comet analysis. One hundred individual cells (50 cells from each slide in duplicate) were randomly analyzed per sample.

**Statistical analysis.** The data were analyzed using the statistical software (Jandel Sigmastat, 2.03). The results were expressed as mean±SEM. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey test. *P<0.05* was considered to be statistically significant.

**Results**

**Effects of ASA and TSA co-treatment on MMC-induced DNA damage.** The various parameters of the comet assay in bone marrow cells of mice treated with ASA and TSA (5, 10 or 20 mg/kg body weight) for 3 and 7 consecutive days are presented in Tables I and II respectively. The observed tail length (TL), tail moment (TM) and percentage DNA in tails (TDNA) in MMC-treated animals were significantly (*P<0.001*) higher than in the negative controls. All of the tested doses of ASA as well as TSA were effective in exerting significant protection against the genotoxicity of MMC as evident from a reduction in the observed TL, TM and TDNA, compared to the positive control group.

**Discussion**

The results reported here demonstrate the anti-genotoxic potential of ASA and TSA. Co-treatment of TSA at the tested doses significantly reduced the DNA damage induced by MMC in bone marrow cells as indicated by the decrease in the comet parameters TL, TM and TDNA, as well as a decrease in the frequency of MNRETs in peripheral blood.

MMC is an extensively studied genotoxic agent and recommended as a reference positive mutagen in genotoxicity studies by the Organization for Economic Co-operation and Development (11, 12). It is a prototype bioreductive alkylating agent which is enzymatically reduced in cells, forming different DNA adducts and yielding reactive species that are capable of producing radicals through reduction cycles (13-16). Metabolism of MMC leads to generation of mono- and bi-functional alkylating species and/or reactive oxygen species (ROS). DNA alkylation results in both inter- and intra-strand cross-linking of two nucleic acid chains or linking nucleic acid to protein, which could be a major factor for the disruption of nucleic acid functions. Cross-linking of DNA prevents the action of vital DNA processing enzymes and inhibits DNA synthesis. Specific cellular responses with respect to DNA damage include cell-cycle arrest, perturbation in DNA repair and apoptosis, which could be a major factor for the disruption of nucleic acid functions. Cross-linking of DNA prevents the action of vital DNA processing enzymes and inhibits DNA synthesis. Specific cellular responses with respect to DNA damage include cell-cycle arrest, perturbation in DNA repair and apoptosis, which could be a major factor for the disruption of nucleic acid functions. Cross-linking of DNA prevents the action of vital DNA processing enzymes and inhibits DNA synthesis. Specific cellular responses with respect to DNA damage include cell-cycle arrest, perturbation in DNA repair and apoptosis, which could be a major factor for the disruption of nucleic acid functions.
thiosulphate and N-acetyl-cysteine are reported to ameliorate anticancer drug-induced oxidative DNA damage and cellular injury (4, 5). TSA may be a promising chemoprotective agent.

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References

Figure 1. Protective effect of ASA and TSA against MMC-induced micronucleus frequency. ASA and TSA reduced MMC-induced frequency of micronucleated reticulocytes (MNRETs) in a dose-dependent manner in the 3- (A) and 7-day (B) study protocols. The individual data of the frequency of MNRETs in different groups in the 3- and 7-day study protocols are shown in (C) and (D) respectively. All the values are shown as mean±SEM (n=5), ***p<0.001, a vs. Control and b vs. MMC alone-treated group (one-way ANOVA followed by the Tukey test).