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RESEARCH ARTICLE

Efficacy of acetylsalicylic acid (aspirin) in skin B16-F0 melanoma tumor-bearing C57BL/6 mice

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Abstract Several epidemiological studies show that aspirin can act as a chemopreventive agent and decrease the incidences of various cancers including melanoma. In this work, we investigated the in vitro and in vivo efficacy of acetylsalicylic acid (ASA) as an antimelanoma agent in B16-F0 cells and skin B16-F0 melanoma tumor mouse model. Our findings indicate that the IC_{50} (48 h) for ASA in B16-F0 melanoma cells was 100 μM and that ASA caused a dose- and time-dependent GSH depletion and increase in reactive oxygen species (ROS) formation in B16-F0 melanoma cells. Male C57BL/6 mice were inoculated s.c. with 1×10^6 B16-F0 melanoma cells. ASA (80, 100, and 150 mg/kg) was initiated on day 1 or day 7, or day 9 after cell inoculation and continued daily for 13, 7, and 5 days, respectively. Animals were weighed daily and sacrificed on day 13. The tumors were excised and weighed. The animals receiving 13 days of ASA therapy at 80, 100, and 150 mg/kg demonstrated tumor growth inhibition by 1±12 %, 19±22 %, and 50±29 %, respectively. Animals receiving 7 days of therapy at 80, 100, and 150 mg/kg demonstrated tumor growth inhibition by 12±14 %, 27±14 %, and 40±14 %, respectively. No significant tumor growth inhibition was observed with 5 days of therapy. ASA at 100 and 150 mg/kg caused significant tumor growth inhibition in C57BL/6 mice when administered for 13 and 7 days, respectively. The results obtained in this study are consistent with the recent epidemiologically based report that aspirin is associated with lower melanoma risk in humans.

Keywords Aspirin · Melanoma · Tyrosinase · ASA · B16-F0 · C57BL/6 mice

Abbreviations

ASA	Aspirin, acetylsalicylic acid
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
TBARS	Thiobarbituric acid-reactive substances
ALT	Alanine aminotransferase

Introduction

Melanoma is one of the most aggressive cancers with relatively high propensity for metastasis [1]. The FDA-approved chemotherapy for treatment of metastatic disease has to date resulted in little or no impact on survival, even when assessed in combination with therapeutics with diverse mechanisms of action [2]. An improvement in outcome for patients with metastatic and primary disease through novel chemotherapeutic agents is urgently needed. Tyrosinase has previously been used as a molecular target in melanoma-directed enzyme prodrug therapy [3–6]. The presence of tyrosinase in human melanomas has led to a rational prodrug approach targeted towards this disease [6–9]. In our studies, tyrosinase was used as a molecular target in an effort to develop antimelanoma drugs that could be bioactivated intracellularly to selectively target malignant melanoma cells while sparing other normal tissues such as the liver and kidney devoid of the enzyme from

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unwanted drug toxicity. Recently, we investigated the in vitro toxicity of ASA in melanoma and non-melanoma cell lines [8]. The study suggested that ASA is selectively more toxic towards five melanocytic melanoma cells (SK-MEL-28, SK-MEL-5, MeWo, B16-F0, and B16-F10), which express functional tyrosinase compared to four non-melanoma cells (SW-620, Saos-2, BJ, and PC-3) and two amelanotic melanoma cells (SK-MEL-24 and C32), which do not express functional tyrosinase [8].

Regular use of acetylsalicylic acid (aspirin, ASA) has been shown to decrease the risk of a variety of cancers including the colon, breast, lung, liver, and prostrate tissues [10–13]. A more recent epidemiological study showed that people who reported using aspirin for 5 years or more had a 20 % lower risk of developing melanoma [14]. The antitumor properties of ASA were ascribed to its COX-2 inhibitory activity [15]. However, results from several studies suggest that the wide range of antiproliferative activities of ASA do not correlate exclusively with its COX-2 inhibitory activity [15–17]. Consequently, in the current work, for the first time, we investigated the in vivo efficacy of ASA as an antimelanoma agent in the B16-F0 skin melanoma tumor model in C57BL/6 mice. Our findings suggest that ASA may have an antimelanoma effect in tumor-bearing mice.

Materials and methods

Materials

All materials, solvents, and reagents used in this work were of analytical grade with the highest degree of purity and were purchased either from Sigma-Aldrich, St. Louis, MO or Fisher Scientific, Pittsburgh, PA. Picric acid (cat. no. 5860-16) was obtained from Ricca Chemical, Arlington, TX.

Cell lines and culture conditions

Dulbecco's Modified Eagle Medium (DMEM) (1×) (GIBCO cat. no. 11965), fetal bovine serum (FBS) (cat. no. 10082-139), penicillin-streptomycin (10,000 U/mL) (cat. no. 15140-122), and trypsin-EDTA solution 1× (0.25 % *w/v*-0.53 mM EDTA) (cat. no. 30-2101) were purchased from American Type Culture Collection (ATCC), Manassas, VA. The B16-F0 (ATCC no. CRL-6322) cell line was obtained from ATCC, Manassas, VA.

Cell culture

The cell culture experiments were performed as described previously [8, 18, 19]. B16-F0 melanoma cells contained in frozen vials were washed twice with DMEM media before culturing in 10 mL media supplemented with (10 %) FBS and streptomycin/penicillin (100 U/mL) in a T-25 flask [19, 20]. The cultures were kept at 37 °C under 5 % CO_2 atmosphere in a TS Autoflow CO_2 Water-Jacketed Incubator (Forma Scientific, Marietta, OH, USA). Upon attainment of ~90 % confluency, the media was removed and trypsin-EDTA solution (2–5 mL) was used to detach cells. The detached cells were rinsed with ~10 mL of prewarmed sterile phosphate-buffered saline (PBS) at 37 °C to dilute trypsin. The mixture was transferred into a 50-mL tube. The flask was additionally rinsed with sterile PBS, and the contents were added to the rest of the cells collected. The cells were spun down at 800 rpm (Beckman GPR Centrifuge, Fullerton, CA) for 3–5 min. Pelleted cells were resuspended in DMEM media supplemented by (10 %) FBS and streptomycin/penicillin (100 U/mL) followed by transferring the mixture into one T-75 flask containing 33 mL media (25 % of the media was supplemented from the previous culture step as conditioning media). After reaching a confluency of 90 %, the cells obtained from each flask were further split into three T-75 flasks in a similar manner [18].

Cell viability determination in murine B16-F0 melanoma cells

Cell viability was determined as described previously [21]. For instance, B16-F0 cells obtained from each flask were suspended in 4 mL of DMEM media supplemented by (10 %) FBS and streptomycin/penicillin (100 U/mL). The cells were then counted using the trypan blue exclusion method for determining the viability [21, 22].

IC_{50} (48 h) determination in B16-F0 melanoma cells

The required concentration (IC_{50} in micromolar) of the compound that can lead to a 50 % decrease in B16-F0 melanoma cell viability 48 h after incubation was calculated from the logarithmic regression equation derived from graphing the viability of the cells at 48 h versus the concentration of the drug. The ASA at calculated concentration was tested to confirm the IC_{50} at 48 h as described above.

Intracellular GSH measurement

Intracellular GSH was determined in murine melanocytic B16-F0 melanoma cells based on a modified recycling method [7, 19, 23, 24]. In this reaction, GSH reacts with DTNB to form the disulfide GS-TNB, and the yellow-colored compound 5-thio-nitrobenzoic acid (TNB) was measured [7, 19, 23, 24].

Briefly, murine melanocytic B16-F0 cells were obtained from exponentially growing 90–95 % confluent cultures and seeded at 1 million cells/mL in DMEM media supplemented with (10 %) FBS and streptomycin/penicillin (100 U/mL) in 24-well plates. The cells were incubated at 37 °C for 3 h to allow for cell adhesion and environmental adaptation. Subsequently, the cells were treated with an additional 1 mL

DMEM media containing various concentrations of ASA (100 μM–1 mM) for 1, 2, and 3 h, respectively. For each time point, the medium was removed and 100 μL trypsin-EDTA solution was added to dislodge the cells, followed by the addition of 100 μL and 3 % sulfosalicylic acid (SSA) to lyse the cells. The cells were monitored under a microscope in order to ensure that all cells were detached and in suspension. The cells were then centrifuged at 14,000 rpm (4 °C) for 10 min. The supernatant was diluted 10-fold with phosphate buffer (100 mM containing EDTA 1 mM), pH 7.4. An aliquot of 50 μL was added to respective wells in a 96-well plate. To each well, 100 μL of master mix (NADPH 0.3 mM, DTNB 0.225 mM, GSSG reductase 1.6 U/mL in phosphate buffer 100 mM containing EDTA 1 mM, pH 7.4) was added. Immediately upon addition of the master mix, color development was recorded at 405 nm at nine time intervals of 30 s for 4 min using a Wallac 1420 microplate reader (PerkinElmer, Wallac, Turku, Finland) equipped with kinetic analysis software [7, 8].

Reactive oxygen species formation measurement

Reactive oxygen species (ROS) formation was determined in murine melanocytic B16-F0 melanoma cells as described previously [7, 23]. Murine melanocytic B16-F0 cells were seeded at 50,000 cells per well in 96-well black microplates (cat. no. 3603, Corning Incorporated, Corning, NY). The cells were incubated at 37 °C for 2 h to allow for cell adhesion and environmental adaptation. The media was removed followed by the addition of 100 μL of 2′,7′-dichlorofluorescein diacetate (10 μM) [25]. Various concentrations of ASA (50–500 μM) were added to the wells. Immediately upon addition, the plates were read at λ_{em}=535 nm using λ_{ex}=485 nm over a period of 5–40 min by a SPECTRAFluor Plus plate reader (Tecan Inc., Santa Margarita, CA).

Animal housing and protocol

Male C57BL/6 mice (6–8 weeks old) were obtained from Charles River Laboratories, Wilmington, MA, fed ad libitum, and allowed to acclimatize for 1 week on clay chip bedding in a room with a 12-h light photo cycle, an environmental temperature of 21–23 °C, and 50–60 % relative humidity. The animal protocols used in the current investigation were reviewed and approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center, Amarillo, TX.

Tumor cell inoculation and administration of drug

Male C57BL/6 mice (6–8 weeks old, n=9) were obtained from Charles River Laboratories, Wilmington, MS. Mice were inoculated [26] subcutaneously on the shaved lateral right flank with one million B16-F0 murine melanoma cells in 100 μL PBS, pH=7.4 using a 25-gauge needle (Becton Dickinson, cat. no. 305122, Franklin Lanes, NJ). Animals were randomized and assigned to different treatment groups with different doses of ASA (80, 100, and 150 mg/kg/day in 100 μL PBS), respectively. Therapy was initiated either on day 1 or day 7 or day 9 post-tumor cell inoculation and continued until day 13 and henceforth referred to as 13, 7, and 5 days of ASA therapy, respectively.

ASA in vivo efficacy study

Treatment of B16-F0 tumor-bearing mice (n=9) was initiated 1 day post-melanoma cell inoculation. The treatment regimen consisted of a daily intraperitoneal injection of ASA injected at doses of 80, 100, and 150 mg/kg in 100 μL PBS. Untreated tumor-bearing mice received 100 μL PBS alone. The animals were weighed on a daily basis. All animals were sacrificed 2 h post-drug injection on day 13 after melanoma cell inoculation; subcutaneous tumors were excised and weighed.

Plasma alanine aminotransferase (ALT) levels, the extent of lipid peroxidation in the liver and kidney as measured by thiobarbituric acid-reactive substances (TBARS) assay and free thiol content in the liver and kidney, were measured in the liver and kidney of C57BL/6 mice as the indication of in vivo toxicity. The daily treatment was initiated on day 1, day 7, and day 9 post-tumor cell inoculation and lasted for 13, 7, and 5 days, respectively.

In vivo toxicology

Plasma ALT levels

On day 13 after tumor cell inoculation, animals were anesthetized using ~2–3 % isoflurane 2 h post-drug injection. Blood samples were drawn via heart tap into tubes with heparin (Baxter Healthcare Corporation; 10,000 USP units/mL; NDC no. 0641-0410-25; 1 part heparin and 9 parts blood). Blood plasma was separated from whole blood by centrifugation at 3,000 rpm (E.I. Du Pont de Nemours & Co., Newtown, CT; cat. no. RC-5C) for 5 min at 4 °C. Plasma ALT levels were determined using Biotron Assay Kit (cat. no. 68-D, Hemet, CA).

Estimation of free thiol content in the liver and kidney of mice

Total sulfhydryl groups were determined by a modified method of Sedlak et al. [27]. Liver and kidney tissues from melanoma tumor-bearing mice were isolated. Tissue of 150–200 mg was mixed with 10 fold of (1 %*w/v*) picric acid and homogenized using a handheld homogenizer. Aliquots of tissue homogenates of 600 μL were then mixed with 60 μL

of (30 %) trichloroacetic acid. The samples were then centrifuged at 7,500 rpm (Brinkmann Instruments Inc., cat. no. 5417C, Westbury, NY) for 10 min. A 100 μL aliquot of the supernatant was then added to a mixture of Ellman's reagent (DTNB) (100 μL; 6 mM) and phosphate buffer (800 μL; 0.1 M, pH 7.4, 1 mM DETAPAC). The absorbance of the solution was observed at 412 nm. The baseline absorbance was corrected with a mixture of 100 μL Ellman's reagent (DTNB) (6 mM) and 900 μL phosphate buffer (0.1 M, pH 7.4, 1 mM DETAPAC) as a blank solution.

Estimation of lipid peroxidation in the liver and kidney of mice

TBARS were measured spectrophotometrically as described by Wills [28, 29]. The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of TBARS. Briefly, 150–200 mg of tissue was mixed with 10-fold distilled water and homogenized using a handheld homogenizer. Liver or kidney tissue homogenate of 0.5 mL was mixed with an equal volume of Tris/HCl buffer (pH 7.4) and was incubated at 37 °C in a water bath for 2 h. The blank solution contained a mixture of 0.5 mL distilled water and 0.5 mL Tris/HCl buffer (pH 7.4), which was used for correction of baseline at 540 nm. The reaction mixture was brought to 2 mL with 1 mL of 10 % ice-cold trichloroacetic acid. After centrifugation (5,000 g, 10 min), 1 mL of 0.67 % thiobarbituric acid (Sigma-Aldrich, St. Louis, MO; cat. no. T5500) was added to 1 mL of the supernatant. The tubes were then kept in a boiling water bath for 10 min, and after cooling under tap water, 1 mL of distilled water was added. Absorbance was measured at 540 nm.

Statistical analysis

Statistical analysis of the data was performed using the post *t* test after ANOVA and was considered to be significant at $p<0.05$.

Results

IC_{50} (48 h) in B16-F0 melanoma cells

The IC_{50} (48 h) concentration was determined by MTT assay [18, 20] as a measure of melanoma cell viability. Varying concentrations of ASA were tested repeatedly to determine B16-F0 melanoma cell toxicity at 48 h. ASA IC_{50} of 100 μM was initially determined using regression analysis of ASA toxicity at various concentrations. The derived value (100 μM) was then confirmed experimentally by repeated testing at 100 μM concentration. The regression analysis of the toxicity of ASA at various doses showed the ASA cytotoxicity to be dose and time dependent (data not shown).

Intracellular GSH measurement in murine B16-F0 melanoma cells

The effect of ASA on intracellular GSH depletion was investigated in murine B16-F0 melanoma cells. Our findings indicate that ASA demonstrated a dose- and time-dependent decline in intracellular GSH levels in murine B16-F0 melanoma cells. For instance, ASA (100 μM, 250 μM, 500 μM, 1 mM) depleted 17, 45, 49, and 63 % of intracellular GSH at 2 h incubation with murine B16-F0 melanoma cells. ASA (250 μM) depleted 15, 45, and 53 % of intracellular GSH at 1, 2, and 3 h incubation time (Fig. 1a).

To ensure that the depletion in the GSH intracellular level was due to the direct ASA-induced cell toxicity but not a change in cell viability, the intracellular GSH was not measured beyond 3 h after the addition of the ASA. This is because a significant drop in cell viability was observed 3 h after incubation with cells as measured by MTT assay [18, 20] when one million cells were seeded per well in a 24-well plate (data not shown). One million cells were used to enhance the analytical accuracy and sensitivity of the measurement.

Reactive oxygen species formation in B16-F0 melanoma cells

ROS formation was investigated using the 2′,7′-dichlorofluorescein assay [25] when ASA was incubated with murine B16-F0 melanoma cells. Our results indicate that ASA at concentrations ranging from 100 μM to 1 mM showed a dose- and time-dependent increase in ROS formation in murine melanocytic B16-F0 melanoma cells (Fig. 1b). For instance, at 40 min incubation time, ASA at 100 μM, 250 μM, 500 μM, and 1 mM showed a 35-fold, 38-fold, 43-fold, and 47-fold increase in ROS formation when incubated with murine B16-F0 melanoma cells (Fig. 1b).

ASA in vivo efficacy study

The in vivo efficacy of ASA was evaluated at 80, 100, and 150 mg/kg/day in a skin melanoma tumor model of C57BL/6 mice [26]. The dosing period varied depending on the tumor growth rate and generally covered the exponential tumor growth period. The results of representative studies of different time points are summarized in Table 1. Figure 2a–c shows the dose-dependent efficacy of ASA against B16-F0 murine melanoma when tumor-bearing mice were treated at different doses (80, 100, and 150 mg/kg) daily for 13, 7, and 5 days, respectively. When compared to PBS-treated controls, the animals receiving 13 days of ASA (80, 100, and 150 mg/kg, i.p.) therapy demonstrated tumor growth inhibition by 1± 12 %, 19±22 %, and 50±29 %, respectively (Fig. 2a). Similarly, when compared to PBS-treated controls, the

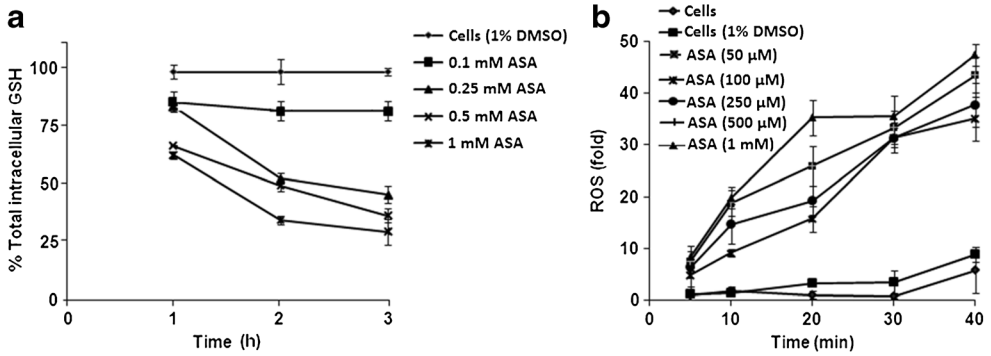

Fig. 1 Intracellular GSH depletion and ROS formation in murine melanocytic B16-F0 melanoma cells. **a** Intracellular GSH depletion by ASA in murine melanocytic B16-F0 melanoma cells, ASA (100 μM–1 mM) showed dose- and time-dependent intracellular GSH depletion in murine melanocytic B16-F0 melanoma cells, which express functional tyrosinase. **b** ROS formation in murine melanocytic B16-F0 melanoma cells; ASA (100 μM–1 mM) led to a dose- and time-dependent increase in ROS formation in murine melanocytic B16-F0 melanoma cells

animals receiving 7 days of ASA (80, 100, and 150 mg/kg) therapy demonstrated tumor growth inhibition by 12±14 %, 27±14 %, and 40±14 %, respectively (Fig. 2b). Animals receiving 5 days of ASA (80, 100, and 150 mg/kg) therapy demonstrated tumor growth inhibition by 0±10 %, 0±11 %, and 2±6 %, respectively (Fig. 2c).

The average tumor size for control mice receiving 100 μL PBS alone was 2,700 mg, while the average tumor size in mice receiving 80, 100, and 150 mg/kg ASA therapy for 13 days was 2,686, 2,187, and 1,347 mg, respectively (Table 1). In mice receiving ASA therapy for 7 days, the average tumor size for control mice receiving 100 μL PBS alone was 2,502 mg, while the average tumor size in mice receiving 80, 100, and 150 mg/kg ASA therapy for 7 days was 2,201, 1,826, and 1,505 mg, respectively (Table 1). For mice receiving 5 days of ASA therapy, the average tumor size for control mice receiving 100 μL PBS alone was 2,727 mg, while the average tumor size in mice receiving 80, 100, and 150 mg/kg ASA therapy for 5 days was 2,714, 2,708, and 2,670 mg, respectively (Table 1).

There are two methods which are generally used to evaluate tumor size in the skin B16-F0 melanoma tumor model: (1) tumor volume using calipers and standard formulas or (2) tumor weight after s.c. tumor excision as an end point [30]. However, tumor volume measurement is not considered as a reliable assessment due to high variability between observers, observer technique, and equipment errors. Hence, tumor excision was used as the end point in our study because it is considered a more accurate measurement of tumor size.

In summary, a comparison among the three doses of aspirin showed that an increase in the strength of the dose led to a corresponding increase in efficacy. For instance, 7 days of ASA therapy at 80, 100, and 150 mg/kg/day demonstrated tumor growth inhibition by 12±14 %, 27±14 %, and 40±14 %, respectively. A comparison among the three durations of therapy showed that the 7-day therapy initiated on day 7 post-cell inoculation showed a better response. For instance, ASA (150 mg/kg/day) therapy for 5, 7, and 13 days demonstrated tumor growth inhibition by 2±6 %, 40±14 %, and 50±29 %, respectively.

In vivo toxicology

In parallel with the in vivo efficacy evaluation, the animal weights were measured on a daily basis. In addition, plasma samples and liver and kidney tissues from respective treatment groups of melanoma tumor-bearing mice were obtained for toxicological analysis.

For analysis of weight change, the animal weights were first corrected by subtracting the tumor weight from the total weight of the animal. The statistical analysis results indicate no significant difference between animal weights of drug-treated mice and control mice, except for animals receiving ASA (150 mg/kg, treated for 13 and 7 days) in comparison to the controls receiving PBS. Mice that received ASA (150 mg/kg) for 7 or 13 days had significantly greater weight for an average of 1.8±0.7 g (10±4 % increase in weight) and 2.7±0.5 g (13±2 % increase in weight) than the animals in the tumor-bearing control group, respectively.

Assessment of liver toxicity

Analysis of blood samples obtained by heart tap revealed that there were significant differences in the plasma ALT level of the PBS-treated control mice and the mice receiving 13 days ASA therapy (80, 100, and 150 mg/kg) (Table 1). The average ALT values in control mice receiving 100 μL PBS alone for 13 days of therapy was 72±6 IU/L, while in mice receiving ASA 80, 100, and 150 mg/kg, the average values were 93±10 IU/L, 155±13 IU/L, and 283±16 IU/L, respectively. In mice receiving ASA (80, 100, and 150 mg/kg) therapy for 7 days, the ALT values were 80±14 IU/L, 111±21 IU/L, and 144±27 IU/L, respectively. The average ALT value for control mice was 64±5 IU/L. In mice receiving ASA (80, 100,

Table 1 Summary of ASA in vivo toxicological and efficacy in melanoma tumor-bearing mice

	Treatment (i.p. injection)			
	Control	80 mg/kg/day	100 mg/kg/day	150 mg/kg/day
13 days ASA therapy				
Average tumor weight (mg)	2,700±401	2,686±325	2,187±588	1,347±798
Tumor burden (%)	100±15	99±12	81±22	50±29[a]
Plasma ALT levels (IU/L)	72±6	93±10	155±13[a]	283±16[a]
Free thiol content (%)				
Liver	100±8	83±3	68±3[a]	46±5[a]
Kidney	100±8	82±4	64±4[a]	48±5[a]
Elevation in lipid peroxidation (%)				
Liver	100±10	127±7	185±14[a]	302±31[a]
Kidney	100±12	128±6	188±11[a]	314±19[a]
7 days ASA therapy				
Average tumor weight (mg)	2,502±498	2,201±500	1,826±498	1,505±501
Tumor burden (%)	100±14	88±14	73±14[a]	60±14[a]
Plasma ALT levels (IU/L)	64±5	80±14[a]	111±21[a]	144±27[a]
Free thiol content (%)				
Liver	100±8	85±3	77±5[a]	64±4[a]
Kidney	100±9	84±3	77±3[a]	74±4[a]
Elevation in lipid peroxidation (%)				
Liver	100±8	119±7	139±16	166±18[a]
Kidney	100±8	127±16	131±12	163±20[a]
5 days ASA therapy				
Average tumor weight (mg)	2,727±204	2,714±266	2,708±293	2,670±153
Tumor burden (%)	100±8	100±10	100±11	98±6
Plasma ALT levels (IU/L)	70±9	77±10	81±13[a]	98±15[a]
Free thiol content (%)				
Liver	100±9	90±8	79±2[a]	63±4[a]
Kidney	100±10	86±3	74±4[a]	60±4[a]
Elevation in lipid peroxidation (%)				
Liver	100±8	139±9	172±10[a]	160±21[a]
Kidney	100±9	150±14[a]	184±19[a]	174±16[a]

Male C57BL/6 mice (6–8 weeks, $n=9$) were inoculated s.c. (day 0) on the right flank with 1×10^6 B16-F0 melanoma cells in 100 μL PBS. ASA (80, 100, and 150 mg/kg/day in 100 μL PBS) was administered i.p. on day 1, day 7, and day 9 post-melanoma cell inoculation and continued daily for 13, 7, and 5 days, respectively. The non-treated control animals received 100 μL PBS only. All animals were weighed daily and sacrificed 2 h post-drug injection on day 13 after inoculation. No significant difference in body weight was observed between control mice and drug-treated mice, except for animals receiving ASA (150 mg/kg and treated for13 and 7 days) in comparison to control mice that received PBS. Plasma ALT levels, lipid peroxidation, and free thiol content of the liver and kidney were measured as biomarkers of ASA-induced in vivo toxicity. Marginal in vivo toxicity was observed when ASA was administered at 80 mg/kg/day i.p., while ASA at 100 and 150 mg/kg caused a significant elevation in lipid peroxidation and depletion in free thiol content in the liver and kidney of melanoma tumor-bearing mice, respectively, when compared to controls

[a] Significantly different

and 150 mg/kg) therapy for 5 days, the ALT values were 77± 10 IU/L, 81±13 IU/L, and 98±15 IU/L, respectively, while the average ALT value for control mice in this group was 70±9 IU/L.

A comparison among the study groups shows that an increase in duration and dose of ASA therapy led to a corresponding increase in toxicity.

Estimation of free thiols in the liver and kidney

No significant depletion in free thiol content was found in the liver and kidney homogenates of B16-F0 tumor-bearing mice treated with ASA (80 mg/kg) (Table 1), irrespective of whether therapy was given for 13, 7 or 5 days. Mice receiving ASA (100 and 150 mg/kg) daily

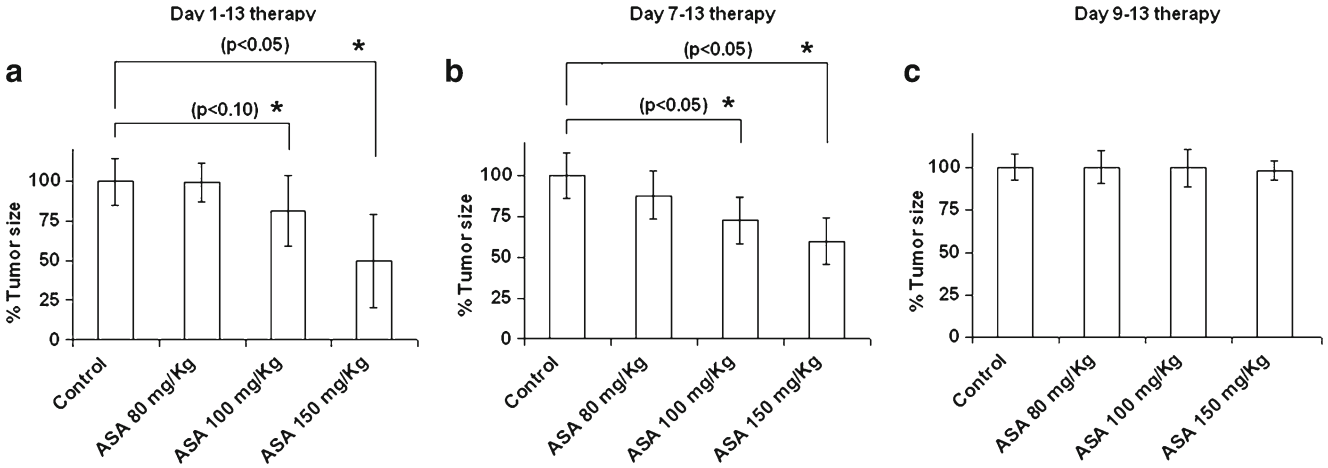

Fig. 2 ASA in vivo efficacy in melanoma tumor-bearing mice. **a** When compared to controls, the animals (n=9) receiving 13 days of ASA therapy (80, 100, and 150 mg/kg in PBS, i.p.) demonstrated tumor growth inhibition by 1±12 %, 19±22 %, and 50±29 %, respectively. **b** Animals (n=9) receiving 7 days of ASA therapy (80, 100, and 150 mg/kg in PBS, i.p.) demonstrated tumor growth inhibition by 12±14 %, 27±14 %, and 40±14 %, respectively, when compared to controls. **c** Animals (n=9) receiving 5 days of ASA therapy (80, 100, and 150 mg/kg in PBS, i.p.) demonstrated tumor growth inhibition by 0±10 %, 0±11 %, and 2±6 %, respectively, when compared to controls. Control mice received PBS alone

therapy for 13 days demonstrated 32±3 % and 54±5 % depletion of free thiol content in the liver homogenate, respectively, and 36±4 % and 52±5 % depletion of free thiol content in the kidney homogenate of C57BL/6 mice, respectively, in comparison to the liver and kidney homogenates obtained from PBS-treated control animals (Table 1).

Mice receiving ASA 100 and 150 mg/kg daily therapy for 7 days demonstrated 23±5 % and 36±4 % depletion in free thiol content in the liver homogenate, respectively, and 23±3 % and 26±4 % depletion of free thiol content in the kidney homogenate of C57BL/6 mice, respectively, in comparison to the liver and kidney homogenates obtained from PBS-treated control animals.

Mice receiving ASA 100 and 150 mg/kg daily therapy for 5 days demonstrated 21±2 % and 37±4 % depletion in free thiol content in the liver homogenate, respectively, and 26±4 % and 40±4 % depletion of free thiol content in the kidney homogenate of C57BL/6 mice, respectively, in comparison to the liver and kidney homogenates obtained from PBS-treated control animals.

Estimation of free thiol content in the liver and kidney homogenates also showed a corresponding increase in toxicity with an increase in the duration and dose of ASA therapy.

Estimation of lipid peroxidation in the liver and kidney

Malondialdehyde (MDA), assayed in the form of TBARS, was used as a measure of lipid peroxidation [28, 29]. No significant increase in MDA concentration was found in the liver and kidney homogenates of B16-F0 tumor-bearing mice treated with ASA (80 mg/kg) (Table 1), irrespective of whether therapy was given for 13, 7, or 5 days. Significant increase in MDA concentration was found in the liver and kidney homogenates of the mice treated with ASA (100 and 150 mg/kg) (Table 1).

Mice receiving ASA 150 mg/kg daily therapy for 13 days demonstrated 302±31 % and 314±19 % elevation in lipid peroxidation in the liver and kidney homogenates of C57BL/6 mice, respectively, in comparison to PBS-treated control animals. The average MDA concentration in the liver and kidney of control mice that received 100 μL PBS alone was 204±20 μM and 200±24 μM, respectively.

ASA (150 mg/kg) demonstrated 166±18 % and 163±20 % elevation in lipid peroxidation in the liver and kidney homogenates of C57BL/6 mice receiving 7 days of ASA therapy, respectively, in comparison to PBS-treated control animals (Table 1). The average MDA concentration in the liver and kidney of control mice that received 100 μL PBS alone was 196±16 μM and 198±16 μM, respectively.

When ASA treatment was given daily for 5 days, ASA (150 mg/kg) demonstrated 160±21 % and 174±16 % elevation in lipid peroxidation in the liver and kidney homogenates of C57BL/6 mice, respectively, in comparison to PBS-treated control animals. The average MDA concentration in the liver and kidney of control mice that received 100 μL PBS alone was 184±15 μM and 188±17 μM, respectively. The longer the duration of therapy, the greater the increase in MDA content of the liver and kidney homogenates as a measure of lipid peroxidation formation was observed.

Estimation of MDA content also showed a corresponding increase in toxicity with increase in the duration and dose of ASA therapy.

Discussion

In the current work, we investigated intracellular GSH depletion and ROS formation by ASA in murine B16-F0 melanoma cells. Our findings indicate that ASA demonstrated a dose- and time-dependent decline in intracellular GSH level and increase in ROS formation in murine B16-F0 melanoma cells. ASA (100 μM, 250 μM, 500 μM, 1 mM) depleted 17, 45, 49, and 63 % of intracellular GSH at 2 h incubation with murine B16-F0 melanoma cells. A direct relationship between ROS formation and intracellular GSH depletion was observed for murine B16-F0 melanoma cells.

We previously investigated the biochemical mechanism and toxicity of ASA mediated by tyrosinase in human melanoma cell lines [8]. Previously, our investigations indicated that ASA (100 μM, 250 μM, 500 μM, 1 mM) depleted 8, 26, 47, and 69 % of intracellular GSH at 2 h after incubation with human SK-MEL-28 melanoma cells [8]. A direct relationship between ROS formation and intracellular GSH depletion was also observed in SK-MEL-28. The findings indicate that ASA was significantly more toxic towards melanocytic murine B16-F0 and human SK-MEL-28, SK-MEL-5, and MeWo melanoma cell lines that express tyrosinase than non-melanoma and amelanotic C32 melanoma cells, which do not express tyrosinase activity [8, 31, 32]. Our study suggests that tyrosinase-mediated reactive intermediate quinone species formation, intracellular GSH depletion, ROS formation, and induced mitochondrial toxicity significantly contributed towards ASA selective toxicity in human and murine melanocytic melanoma cells [8]. A schematic representation of the molecular events leading to aspirin's anticancer effects is shown in Fig. 3.

Aspirin is a non-steroidal, anti-inflammatory drug (NSAID) extensively used for its analgesic, antipyretic, and antirheumatic properties [33]. Human epidemiological studies and evidence from animal models and in vitro experiments suggest that ASA may suppress tumor progression in colorectal, stomach, and esophageal cancers [10, 11, 15]. The antitumor properties of aspirin were ascribed to its COX-2 inhibitory activity [16, 17]; however, several lines of evidence suggest that the wide range of antiproliferative activities of aspirin do not correlate exclusively with its COX-2 inhibitory activity [17].

Earlier reports on salicylate metabolism indicate a peak plasma level of free salicylate to be 30–60 mg/L (166 −333 μM) for an analgesic dose of aspirin administered for mild aches and pain [34]. Aspirin is also considered the drug of choice for rheumatic diseases, particularly rheumatic fever and rheumatic arthritis, for which up to 10 g/day has been indicated [34]. Plasma salicylate levels in the range of 200–300 mg/L (1.1–1.7 mM) are acceptable for such therapy and are often accompanied by mild toxic manifestations, including tinnitus, gastric disturbances, and anemia [34–36]. Although high-dose aspirin was used in our study, the maximum dose of 150 mg/kg translates to ~10 g dose, which is clinically relevant for high-dose aspirin therapy in rheumatic fever and rheumatic arthritis [34]. In our study, administration of high-dose aspirin (100 and 150 mg/kg) was associated with significant elevation in ALT, MDA content as a measure of lipid peroxidation, and depletion of free thiol content in the liver. This difference in toxicity may be attributed to species difference in humans and mice.

Because ASA demonstrated similar antiproliferative effect, ROS formation and intracellular GSH depletion in both murine melanocytic B16-F0 and human melanocytic SK-MEL-28 melanoma cells [8], we decided to investigate the in vivo efficacy of ASA in a skin tumor model of murine B16-F0 melanoma tumor-bearing C57BL/6 mice before its investigation in melanoma patients. In the current study, treatment of B16-F0 tumor-bearing mice was initiated either on day 1, day 7, or day 9 post-melanoma cell inoculation and continued until day 13, corresponding to 13, 7, and 5 days of ASA therapy, respectively. The different doses and therapy periods were used to study the effects of dose and duration of ASA therapy on the tumor size in the skin B16-F0 melanoma tumor model. ASA dose was selected based on previous studies showing that at a level of 200 mg/kg body weight, this agent is unable to elicit toxicity in Swiss mice [37]. Results from our in vivo efficacy study in B16-F0 melanoma-bearing C57BL/6 mice have showed that administration of ASA (100 and 150 mg/kg/

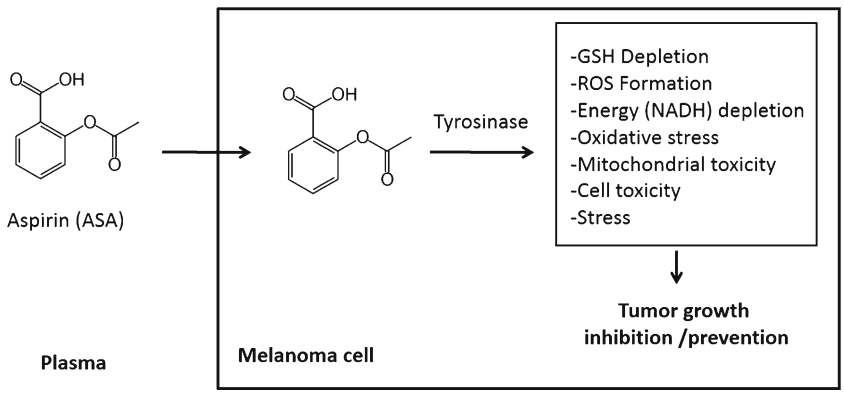

Fig. 3 Schematic representation of the molecular events leading to the anticancer effects of aspirin

day) for 13 or 7 days after melanoma cell inoculation caused significant tumor growth inhibition as measured in the B16-F0 melanoma skin tumor model of C57BL/6 mice, while no significant tumor growth inhibition was observed with 5 days of ASA therapy.

A previous study by Reddy et al. [38] demonstrated that ASA inhibits colon carcinogenesis by 70 % in rats. Another study by Duperron et al. [39] showed that ASA was effective in reducing lung tumorigenesis by 60 %. However, no study investigated the role of ASA in a melanoma tumor model in mice. In the current study, we investigated the in vivo toxicity and efficacy of ASA in B16-F0 melanoma tumor-bearing C57BL/6 mice. Our findings suggest that 7 days of ASA therapy demonstrated the best efficacy with minimal toxicity. Although the exact mechanisms of aspirin-mediated tumor growth inhibition in an animal model have not been established, our cell culture data suggest that growth inhibition may occur via depletion of GSH, ROS generation, and mitochondrial cytotoxicity (Fig. 3).

In summary, the IC_{50} (48 h) for ASA in murine B16-F0 melanoma cells was 100 μM. The animals receiving 7 days of therapy initiated on day 7, post-cell inoculation demonstrated the best response when considering both efficacy and toxicity factors in ASA therapy in the skin tumor model of B16-F0 in C57BL/6 mice. The animals receiving 7 days of ASA (100 and 150 mg/kg) therapy demonstrated significant tumor growth inhibition by 27±14 % and 40±14 %, respectively. Thus, our study shows that aspirin has the potential to be used as an antimelanoma agent and supports a recently published epidemiological report that showed that the use of aspirin may have a chemopreventive effect against the development of melanoma [14].

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Conflicts of interest None

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