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Biochemical mechanism of Acetaminophen (APAP) induced toxicity in melanoma cell lines

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Abstract

In this work, we investigated the biochemical mechanism of acetaminophen (APAP) induced toxicity in SK-MEL-28 melanoma cells using tyrosinase enzyme as a molecular cancer therapeutic target. Our results showed that APAP was metabolized 87% by tyrosinase at 2h incubation. AA and NADH, quinone reducing agents, were significantly depleted during APAP oxidation by tyrosinase. The IC_{50} (48h) of APAP towards SK-MEL-28, MeWo, SK-MEL-5, B16-F0 and B16-F10 melanoma cells was 100μM whereas it showed no significant toxicity towards BJ, Saos-2, SW-620, and PC-3 non-melanoma cells, demonstrating selective toxicity towards melanoma cells. Dicoumarol, a diaphorase inhibitor, and 1-bromoheptane, a GSH depleting agent, enhanced APAP toxicity towards SK-MEL-28 cells. AA and GSH were effective in preventing APAP induced melanoma cell toxicity. Trifluoperazine and cyclosporin A, inhibitors of permeability transition pore in mitochondria, significantly prevented APAP melanoma cell toxicity. APAP caused time and dose-dependent decline in intracellular GSH content in SK-MEL-28, which preceded cell toxicity. APAP led to ROS formation in SK-MEL-28 cells which was exacerbated by dicoumarol and 1-bromoheptane whereas cyslosporin A and trifluoperazine prevented it. Our investigation suggests that APAP is a tyrosinase substrate, and that intracellular GSH depletion, ROS formation and induced mitochondrial toxicity contributed towards APAP's selective toxicity in SK-MEL-28 cells.

Keywords

APAP; melanoma; acetaminophen; SK-MEL-28; MeWo; SK-MEL-5; cancer; quinone

INTRODUCTION

Melanoma is a cancer that arises from melanocytes, specialized pigmented cells that are found predominantly in the skin.1 The incidence of melanoma is rising steadily in western populations;2 the number of cases worldwide has doubled in the past 20 years. In its early stages malignant melanoma can be cured by surgical resection, but once it has progressed to

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the metastatic stage it is refractory to treatment and does not respond to currently available therapies.[1, 3]

4-Hydroxyanisole (4-HA) is a simple phenolic agent which was first shown by Riley to be a melanocytotoxic agent.[4] Tyrosinase, an abundant enzyme found in melanocytes, is used as a selective molecular target for 4-HA bioactivation to cytotoxic o-quinone.[5-7] However, 4-HA clinical trials were terminated because it caused severe liver toxicity.[4,8] The mechanistic pathway of 4-HA metabolism by isolated rat hepatocytes and rat liver microsomes was subsequently investigated.[7] It was found that P450 enzymes play a critical role in the bioactivation of 4-HA to an epoxide and then p-quinone which causes severe liver cell toxicity.[7, 9] A phenolic agent devoid of metabolism by liver P450 enzymes will thereby have minimal liver toxicity.

Acetaminophen (N-acetyl-para-aminophenol,[10] APAP) is a phenolic compound, which was found to be a substrate for tyrosinase. APAP is one of the most extensively used drugs worldwide for its analgesic, antipyretic and anti-inflammatory effects. Earlier, Wolchok et al [11] conducted a phase I trial to investigate if depletion of GSH by APAP would lead to melanoma cell death or increased sensitivity to alkylating agents, or both. Valero et al [12-14] studied the oxidation pathway of APAP to its corresponding o-quinone by tyrosinase. However, none of these studies investigated the biochemical mechanism of APAP toxicity in melanoma cells involving tyrosinase as a molecular therapeutic target.

In this work, for the first time, we have investigated the enzymatic oxidation and the biochemical toxicity of APAP in human SK-MEL-28, MeWo and SK-MEL-5 melanoma cells and murine B16-F0 and B16-F10 melanoma cell lines. We hypothesized that bioactivation of APAP to its corresponding quinone metabolite by melanoma tyrosinase, a molecular target for development of cancer therapeutics, [9] would lead to a selective and desirable melanoma cell death. Cell viability assays were performed to assess the cytotoxicity and anti proliferative effects of APAP towards melanoma cells. A number of biochemical modulators were used to elucidate the mechanism of APAP induced toxicity in SK-MEL-28 human melanoma cells. The implications of intracellular GSH depletion and oxidative stress in mitochondria were also investigated in SK-MEL-28 melanoma cells.

MATERIALS AND METHODS

Materials

All materials, solvents and reagents used in this work were analytical grade with the highest degree of purity and were purchased either from Sigma-Aldrich, St. Louis, MO or Fisher-Scientific, Pittsburgh, PA. Mushroom tyrosinase was used throughout this study because purified human tyrosinase is not available commercially. DMSO was used to dissolve the APAP and modulators for addition to the cell culture. The final concentration of DMSO was 1%, and 2% in cell culture media, which was used in non treated control cells. DMSO was used to solublize APAP and other chemicals. Phosphate buffered saline (PBS) was used as a vehicle to dissolve AA and GSH. Stock solutions of HRP were made in Tris buffer without DETAPAC pH 7.4.

Cell Lines and Culture Conditions

Modified Eagle Medium Alpha (MEM) (1×) (Cat. no. 32571-036), fetal bovine serum (FBS) (Cat. No. 10082-139), Penicillin-Streptomycin (10,000 units/mL), Cat. No. (15140-122) and Trypsin-EDTA solution 1× (0.25%w/v-0.53mM EDTA) (Cat. No. 30-2101) were purchased from American Type Culture Collection (ATCC®), Manassas, VA. RPMI medium 1640 (1×) (Cat. no. 11875-119) was obtained from Invitrogen Corporation, Grand Island, NY. Melanocyte medium (Cat. No. 2201), poly-L-lysine (Cat. No. 0413), trypsin/EDTA solution

(for human epidermal melanocytes primary cells; normal melanocytes) (Cat. no. 0103), and trypsin neutralization solution (Cat. no. 0113) were obtained from ScienCell Research laboratories, Carlsbad, CA. The human SK-MEL-28 melanoma cell line was a gift from Dr. Kelly M. McMasters, Division of Surgical Oncology University of Louisville, KY. The SK-MEL-5 and MeWo melanoma cell lines, Saos-2 osteosarcoma, SW-620 colorectal adenocarcinoma and PC-3 prostate adenocarcinoma cell lines were a gift from Dr. Jon Weidanz, Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, Amarillo, TX. The B16-F0 (ATCC® No. CRL-6322), B16-F10 (ATCC® No. CRL-6475) melanoma and BJ fibroblast cell lines (ATCC® No. CRL-2522) were obtained from ATCC®, Manassas, VA. Human epidermal melanocytes (Catalog no. 2200, ScienCell Research laboratories, Carlsbad, CA) were obtained from ScienCell research laboratories, Carlsbad, CA.

UV-VIS spectroscopy of enzyme mediated metabolism of APAP

A UV-VIS spectroscopy method was used to elucidate the progression of the enzymatic oxidation of APAP by tyrosinase/O_2 and HRP/H_2O_2.9 The spectra of a solution containing APAP (100 μM) and tyrosinase (10 U/mL) or HRP (2 μM)/H_2O_2 (100 μM) were recorded in the absence and presence of GSH (200 μM) using a GBC UV-Visible spectral spectrophotometer, GBC Scientific, Victoria, Australia. The spectra of the mixture were obtained when GSH was added to the solution either before or after the addition of tyrosinase or HRP/H_2O_2. Similarly, the effects of ascorbic acid (AA) (100 μM) and NADH (200 μM) were also investigated. The control spectrum in the absence of tyrosinase or HRP/H_2O_2 was that of the APAP solution (100 μM) and AA (100 μM) or NADH (200 μM) in phosphate buffer 0.1 M (pH 7.4) containing diethylenetriaminepentaacetic acid (DETAPAC) (1 mM). The UV-VIS spectra were taken at 10 min intervals.

Rate of AA and NADH oxidation mediated by APAP metabolism by tyrosinase/O_2 and HRP/H_2O_2

Tyrosinase (5 U/mL) was added to a mixture of APAP (100 μM) in the presence and absence of GSH (200 μM), and AA (50 μM) in phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM).9 A concentration of APAP (100 μM) and tyrosinase (5 U/mL) was used with NADH (200 μM). GSH was measured using Ellman's reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) DTNB.15 The depletion of AA and NADH were monitored at 266 nm and 340 nm respectively. The rate was calculated using the following formula:

Rate of AA oxidation = [change in AA absorbance/initial AA absorbance] × [AA (μM)/APAP(μM)] × [1/tyrosinase(U/mL)] × [1/time(min)]. A similar formula was used to calculate the rate of NADH oxidation. A similar approach was used to measure the rate of AA and NADH oxidation by HRP (2 μM)/H_2O_2 (100 μM).

Tyrosinase mediated GSH depletion assay

A previously described method was used to measure the extent of GSH depletion as a result of the enzymatic oxidation of APAP by tyrosinase/O_2.16 Briefly, tyrosinase (10 μL; 2500 U/mL) was added to a mixture of APAP (100 μM) and GSH (200 μM) in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, containing DETAPAC 1mM). The mixture was pre-incubated for 15 min at 37°C. A 250 μL aliquot was added to trichloroacetic acid (25 μL; 30% w/v), vortexed and left at room temperature for 5 min. A 100 μL aliquot of the supernatant was then added to a mixture of Ellman's reagent (DTNB) (25 μL; 2 mg/mL) and Tris/HCl buffer (875 μL; 0.1 M, pH 8.9), and then vortexed. The absorbance of the solution was observed at 412 nm. 7, 15, 17 GSH depletion was used as a marker of the enzymatic oxidation of APAP by tyrosinase/O_2.

Animal housing and protocol

Adult male Sprague-Dawley rats, 250–300*g*, were obtained from Charles River Laboratories, Wilmington, MA, fed *ad libitum*, were 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 current investigation for rat liver microsomal preparation were reviewed and approved by Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center, Amarillo, TX.

Rat liver microsomal preparation

The CYP2E1 induced microsomes were prepared from rats treated (*i.p.*) with inducing agent pyrazole (200 mg/kg/day) 18 for two consecutive days before sacrificing the rats on the third day. The rats were anesthetized by sodium pentobarbital (60 mg/kg) before surgery in order to prepare the animal before liver removal. The livers were then perfused using 50 mM KCl buffer (pH 7.4). Hepatic microsomes were prepared by differential centrifugation as described previously. 7, 19 Microsomal protein content was determined using the Bio-Rad protein assay kit (Cat. No. 500-0006, Hercules, CA) for protein quantitation.20

Enzymatic Oxidation of APAP by rat liver microsomal preparation

GSH depletion was used to measure the extent of the drug metabolized by rat liver P450 microsomal preparation/NADPH/O_2 system.16 The amount of unreacted GSH was determined colorimetrically using Ellman's reagent (DTNB).17 Incubation mixtures contained in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM), 2 mg/mL pyrazole induced rat liver microsomes, 200 μM GSH, 1 mM NADPH, and 100 μM phenolic agent (4-HA and APAP). The mixtures were gently mixed at 37°C from which 250 μL aliquots were taken at different time points into Eppendorf tubes containing 25 μL trichloroacetic acid (30% w/v). Following protein precipitation and centrifugation for 5 min, the GSH levels of a 100 μL aliquot of the supernatant was determined by the addition of 0.1 M Tris/HCl buffer, pH 8.9 (875 μL), and 2 mg/mL DTNB (25 μL). The reaction product between GSH and DTNB was determined at 412 nm on a GBC spectral spectrophotometer, GBC Scientific, Victoria, Australia.

Cell culture

The cell culture experiments were performed as described previously.16 Human SKMEL-28 melanoma cells contained in frozen vials were washed twice with MEM Alpha media before culturing in 10 mL media supplemented with (10%) fetal bovine serum (FBS), in a T-25 flask.21 The cultures were kept at 37°C under a 5% CO_2 atmosphere in a TS Auto flow CO_2 Water-Jacketed Incubator (Forma Scientific, Marietta, OH, USA). Trypsin-EDTA solution (2-5 mL) was used to detach cells. The detached cells were rinsed with ~10 mL of pre-warmed at 37°C sterile PBS 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, USA) for 3-5 min. Pelletted cells were re-suspended in MEM Alpha media (supplemented by (10%) FBS) followed by splitting 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).16 A similar approach was used to measure cell viability in skin fibroblast BJ cells, prostate PC-3, osteosarcoma Saos-2, colorectal adenocarcinoma SW-620, and melanocytic SK-MEL-5, B16-F0, B16-F10 and MeWo melanoma cells.

Human epidermal primary melanocyte cell culture

Human epidermal primary melanocytes (normal melanocytes) contained in frozen vials were cultured in melanocyte medium supplemented with 0.5% FBS in a T-75 flask which was

previously coated with a polyL-lysine coat (2 μg/cm^2). The T-75 flask was kept at 37°C under a 5% CO_2 atmosphere in a TS Auto flow CO_2 Water-Jacketed Incubator (Forma Scientific, Marietta, OH, USA). The flasks were left undisturbed for at least 16 h initially after the culture was initiated. Growth medium was changed the next day to remove residual DMSO and unattached cells, then every 48 h thereafter. Once the culture attained 50% confluency, media was changed every 24 h until culture was approximately 80% confluent. To detach the cells, 10 ml of PBS diluted trypsin/EDTA solution (1:10) was added for one minute only and removed immediately because the cell are very sensitive to the effect of trypsin. After a 30 seconds wait period, the flask was gently tapped to release the cells from the culture surface. 10 ml of trypsin neutralization solution was immediately added to the flask. The resulting cells were harvested into a 50 ml centrifuge tube and spun down at 1000 rpm (Beckman GPR Centrifuge, Fullerton, CA, USA) for 5 min. Pelletted cells were re-suspended in melanocyte growth medium.

Cell viability

Cell viability was determined as described previously.16 SK-MEL-28 cells obtained from each flask were suspended in 4 mL of MEM Alpha media supplemented by (10%) FBS. The cells were then counted using the trypan blue exclusion method for determining the viability.22

To evaluate cytotoxicity, human SK-MEL-28 melanoma cells were obtained from exponentially growing 90-95% confluent cultures and seeded at 40,000 cells/well in 24-well plates. Human epidermal primary melanocytes, BJ, PC-3, Saos-2, SW-620, B16-F0, B16-F10, and MeWo cells were also seeded at 40,000 cells/well in 24-well plates. SK-MEL-5 cells were seeded at 4,000 cells/well density.

MEM Alpha media was used to culture human SK-MEL-28 cells, SK-MEL-5, MeWo, and BJ cells. B16-F0 and B16-F10 cells were cultured using DMEM media (GIBCO catalog No. 11965). Melanocyte medium (ScienCell catalog no. 2201) was used to culture human epidermal primary melanocytes. The Saos-2 cells were cultured using McCoy's 5A Medium (ATCC® Number: 30-2007) while the SW-620 cells were cultured in Leibovitz's L-15 Medium (ATCC® Number: 30-2008). The PC-3 cells were cultured using RPMI media (Gibco Cat No. 11875-093). The SK-MEL-28 cells were grown in 400 μL fresh MEM Alpha media (supplemented by (10%) FBS). The SK-MEL-28 cells were allowed 24 h for cell adhesion and environmental adaptation. Subsequently, the cells were treated with an additional 600 μL MEM Alpha media containing various concentrations of phenolic agents (4-HA and APAP) for 24 h and 48 h. The medium was removed and the wells were washed twice using MEM Alpha media before adding 160 μL of 2 mg/mL yellow tetrazolium dye (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) (MTT).21 The plates were returned to the incubator for a period of 4 h. The residual MTT solutions were removed from the wells and then 800 μL of DMSO was added to each well. The plates were stored at room temperature in a dark place for an additional 2 h before reading them at 570 nm using SPECTRAFluor Plus plate reader (Tecan Inc, Santa Margarita, CA).

1-Bromoheptane (75 μM), Dicoumarol (10 μM), Ascorbic Acid (AA) (50 μM), and GSH (50 μM) were used as biochemical modulators to asses the mechanism of APAP toxicity in melanoma cells. DMSO was used as a vehicle to dissolve BH, DC, CS and TF. 1% DMSO and 2% DMSO were therefore included as controls in the experiment. PBS was used as a vehicle to dissolve AA and GSH. 1% DMSO was therefore included as a control in the experiment. All experiments were performed in triplicate for three times. An analysis of variance (ANOVA) was carried out to compare the percentage of surviving cells for each compound at various concentrations followed by Bonferoni's post t-test.

IC_{50} calculation

The required concentration of the compound that can cause 50% decrease in melanoma cell viability (IC_{50} in μM) was tested 48 h after incubation with APAP. The linear regression equation was derived after graphing the viability of the cells at 48 h versus the concentration of APAP tested.

Intracellular GSH measurement in human SK-MEL-28 melanoma cells

Determination of intracellular GSH was based on a modified recycling method.23 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.23, 35 Briefly, human SK-MEL-28 were obtained from exponentially growing 90-95% confluent cultures and seeded at 1 million cells/mL in MEM Alpha media 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 MEM Alpha media containing various concentrations of phenolic agents (4-HA and APAP) for 1 and 2 h respectively. For each time point, the medium was removed, 100 μL trypsin-EDTA solution was added to dislodge the cells, followed by addition of 100 μL 3% sulphosalicylic acid (SSA) to lyse the cells. The cells were monitored under the microscope in order to ensure 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. 100 μL of master mix (NADPH 0.3 mM, DTNB 0.225 mM, GSSG reductase 1.6 Units/mL in phosphate buffer 100 mM containing EDTA 1 mM pH 7.4) was added to each well. Immediately upon addition of the master mix, color development was recorded at 405 nm at 9 time intervals of 30 sec for 4 min using a Wallac 1420 microplate reader (Turku, Finland) equipped with kinetic analysis software.35

Intracellular GSH measurement in human epidermal primary melanocytes

A similar procedure to that of SK-Mel-28 was followed for the determination of intracellular GSH in human epidermal primary melanocytes. Briefly, human epidermal primary melanocytes were obtained from exponentially growing 90-95% confluent cultures and seeded at 1.5 million cells/mL in 16 mL melanocyte medium supplemented with 0.5% FBS in a T-75 flask which was previously coated with a poly-L-lysine coat (2 $\mu g/cm^2$). The cells were incubated at 37°C for 16 h to allow for cell adhesion and environmental adaptation. Subsequently, the cells were treated with an additional 4 mL melanocyte medium (supplemented with 0.5% FBS) containing various concentrations of APAP for 1 and 2 h. The medium was removed, 100 μL trypsin-EDTA solution (Cat. no. 0103, ScienCell Research laboratories, Carlsbad, CA) was added to dislodge the cells, followed by addition of 100 μL 3% sulphosalicylic acid (SSA) to lyse the cells. The cells were monitored under the microscope in order to ensure 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. 100 μL of master mix (NADPH 0.3 mM, DTNB 0.225 mM, GSSG reductase 1.6 Units/mL in phosphate buffer 100 mM containing EDTA 1 mM pH 7.4) was added to each well. Immediately upon addition of the master mix, color development was recorded at 405 nm at 9 time intervals of 30 sec for 4 min using a Wallac 1420 microplate reader (Turku, Finland) equipped with kinetic analysis software.23

Reactive oxygen species (H_2O_2) formation in human SK-MEL-28 melanoma cells

SK-MEL-28 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 addition of 100 μL of 2',7'-dichlorofluorescein diacetate.24 Various concentrations of APAP (100-2000 μM) were added to the wells. Immediately upon addition, the plates were read at λ_{em} = 535 nm using λ_{ex} = 485 nm by a SPECTRAFluor Plus plate reader (Tecan Inc, Santa Margarita, CA). Dicoumarol (10 μM), a diaphorase inhibitor,25 1-bromoheptane (75 μM), a GSH depleting agent,26 ascorbic Acid (50 μM), a reducing agent,9 GSH (50 μM), cyclosporin A (15 μM) and trifluoperazine (15 μM), inhibitors of the mitochondrial permeability pore,27 were used as biochemical modulators to asses their effects on ROS formation in melanoma cells.

Computerized time-lapse microscopy in human SK-MEL-28 melanoma cells

Cells were seeded into six-well plates (Falcon, Franklin Lakes, NJ) at a density of 20,000 cells per well. At the start of each experiment, cell cultures were transferred from the incubator to a time-lapse microscope equipped with a heated stage and incubation chamber (Axiovert 200; Zeiss, Gottingen, Germany). The incubation chamber maintained optimum environmental conditions (37°C, 5% CO_2) by independent digital control units (Zeiss, Gottingen, Germany). Three sets of phase contrast images from each of two independent wells were acquired throughout the experiment using a Cohu 2600 Series compact monochrome interline transfer CCD camera, and taken at 300 s intervals. Openlab software automation (Improvision, Lexington, MA) operated the camera, stage movements, and compiled the acquired phase images. Images were then processed as Quicktime movies using the above software and analyzed manually. Every cell in the initial microscopic field was identified and numbered. All identified cells and their progeny were tracked for the duration that they were viewed onscreen. Cells that entered the microscopic field after the initial frame were not included, nor were cells identified as dead at the start of the video. Cells divisions were counted for approximately 100 cells over a 48 h period after drug treatments, with counts being made every 4 h.

Partition coefficient

Partition coefficient values were estimated using the LogP software available at www.logP.com.

RESULTS

UV-VIS spectroscopy of tyrosinase/O_2 and HRP/H_2O_2 mediated enzymatic oxidation of APAP

APAP demonstrates a maximum absorbance peak at 243 nm (Fig. 1A and B). The progression of APAP's enzymatic oxidation was monitored by tyrosinase/O_2 and HRP/H_2O_2 oxidizing systems using a UV-VIS spectroscopy method which demonstrated characteristic peaks at 275-380 nm (Fig. 1A and B). Addition of glutathione at the beginning of the enzymatic oxidation reaction did not result in the formation of the characteristic peaks at 275-380 nm. Ascorbic Acid (AA) and NADH demonstrate distinctive peaks at 266 nm and 340 nm, respectively (Fig. 1A and B). Upon the addition of tyrosinase enzyme, the 266 nm and 340 nm peaks rapidly disappeared and peaks at 275-380 nm developed after complete oxidation of AA and NADH, respectively.

Enzymatic Oxidation of APAP mediated by tyrosinase/O_2

4-HA showed 98% depletion of GSH (at 30 min) which translates to 2 mol GSH per mol of 4-HA. APAP demonstrated 87% depletion which translates to 1.7 mol GSH per mole of APAP after 2 h incubation. Negligible GSH depletion occurred in the absence of the enzyme

(data not shown). The amount of GSH depletion over the indicated incubation times was used to estimate the GSH depletion rate.

The rate of AA and NADH oxidation tyrosinase/O_2 and HRP/H_2O_2

The extent of AA, NADH and GSH depletion were used to measure the extent of APAP enzymatic oxidation by tyrosinase/O_2 and HRP/H_2O_2 (Fig. 2A and B). 9 The order of biochemical depletion by tyrosinase and HRP/H_2O_2 was NADH> AA >>GSH. The order of biochemical depletion when APAP was metabolized by tyrosinase/O_2 was AA>NADH>GSH. GSH was more effective in preventing AA and NADH depletion in tyrosinase/O_2/APAP and HRP/H_2O_2/APAP kinetic reaction.

Enzymatic oxidation of APAP by pyrazole induced rat liver P450 microsomal preparation/ NADPH/O_2 system

APAP showed 33% depletion of GSH at 2 h incubation with CYP2E1 pyrazole induced rat liver microsomes (Fig. 3A), while 4-HA showed 88% depletion (data not shown). On a molar basis, only 0.6 mol GSH was depleted when APAP was metabolized by CYP2E1 pyrazole induced rat liver microsomal preparations after 2 h incubation whereas 4-HA depleted 1.8 mol GSH.

Anti-proliferative assay in melanoma and non-melanoma cell lines

The IC_{50} (48 h) concentration was determined by MTT assay 16, 21 as a measure of SK-MEL-28 melanoma cell viability. The required concentration of the compound that can cause 50% decrease in SK-MEL-28 melanoma cell viability (IC_{50} in μM) was tested at 48 h after incubation with 4-HA and APAP. The phenolic agents (4-HA and APAP) were tested repeatedly to determine melanoma cell toxicity at 48 h after drug addition. The regression analysis of the toxicity of 4-HA and APAP at various doses showed the cytotoxicity to be dose- and time-dependent (data not shown). The cell viability data was determined in the presence and absence of modulators. The modulators were added to the respective wells 20 minutes prior to addition of the phenolic agent. Cell viability was determined 48 h after drug addition. Dicoumarol (10 μM), a diaphorase inhibitor,25 and 1-bromoheptane (BH) (75 μM), an intracellular GSH depleting agent,26 enhanced APAP toxicity. The enhanced effects of dicoumarol on APAP induced toxicity towards melanoma cells were evident at 48 h after incubation (Fig. 4A). DC (10 μM) had negligible effect on APAP (50 μM) toxicity 24 h after incubation with cells (data not shown). At 48 h after incubation, however, DC (10 μM) enhanced APAP (50 μM) toxicity by 25% upon incubation with SK-MEL-28 cells ($P<0.05$) (Fig. 4A). BH (75 μM) increased APAP (50 μM) toxicity by 3% after 24 h incubation with the cells (data not shown). The increase in toxicity was 17% at 48 h after incubation (Fig. 4A) ($P<0.05$). Cyclosporin A (CS), an inhibitor of the permeability transmission pore (PTP) in mitochondria,27 improved cell viability and proliferation at 48 h after incubation in SK-MEL-28 cells (Fig. 4B). 41% viability was observed when CS was co-incubated with APAP as compared to 39% viability when APAP (200 μM) was incubated alone at 48 h post incubation with the drug (Fig. 4B). Similar effects were observed for trifluoperazine (TF) (Fig. 4B). Co-incubation of TF (15 μM), a PTP inhibitor, 27 with APAP (200 μM) increased viability from 39% to 70% at 48 h after incubation in SK-MEL-28 cells (Fig. 4B). APAP toxicity towards SK-MEL-28 melanoma cells was also investigated in the presence and absence of AA and GSH (Fig. 4C). 50% viability was observed when AA (50 μM), a reducing agent,9 was co-incubated with APAP (200 μM) as compared to 38% viability when APAP (200 μM) was incubated alone 48 h after drug addition (Fig. 4C). Similar effects were seen with GSH (Fig. 4C).28 Co-incubation with GSH (50μM) increased cell viability in the presence of APAP (200μM) from 38% to 63% after 48 h of incubation (Fig. 4C). The modulators alone caused no significant effect when incubated alone with the cells.

When tested at 100 μM concentration, APAP showed considerable toxicity in SK-MEL-5, MeWo cells (metastatic human melanoma cell lines) and murine B16-F0 and B16-F10 melanoma cells resulting in 40±3%, 45±7%, 66±8%, and 60±5% cell toxicity respectively (Fig. 5).APAP demonstrated negligible toxicity at 100 μM when tested in prostate (PC-3) cells (viability 90±1%), BJ skin fibroblast cells (viability 91±4%), Osteosarcoma (Saos-2) cells (viability 91±2%) and colorectal adenocarcinoma (SW-620) cells (viability 89±1%) (Fig. 5) demonstrating that APAP was selectively toxic towards the five melanoma cell lines tested in this study. Additionally, when tested at 100 μM concentration, APAP demonstrated 81±2% cell viability in human epidermal melanocytes (Fig. 5). These findings indicate that APAP was significantly more toxic towards melanocytic melanoma cell lines that express tyrosinase than non melanoma cells, which do not express tyrosinase activity while it demonstrated moderate toxicity towards human epidermal primary melanocytes.

Intracellular GSH measurement in SK-MEL-28 melanoma and primary melanocytes

We also studied the effects of intracellular GSH depletion on APAP cytotoxicity in human SK-MEL-28 melanoma cells and human epidermal primary melanocytes at 1 and 2 h post incubation with cells. Our findings indicate that APAP (100, 250, and 500 μM) depleted 7%, 14%, and 21% of intracellular GSH at 1 h and 12%, 22%, and 55% of intracellular GSH 2 h post incubation with human SK-MEL-28 melanoma cells (Fig. 3B). While incubation with human epidermal primary melanocytes (normal melanocyte cells) caused 3%, 6%, and 8% depletion of intracellular GSH at 1 h and 5%, 8%, and 11% at 2 h after incubation with cells (Fig. 3C). Negligible GSH depletion was seen in the absence of APAP. Cell viability was also determined by the trypan blue assay in SK-MEL-28 cells. It was found that APAP at 100 μM, 250 μM and 500 μM caused 9%, 17% and 31% cell death at 2 h (data not shown) indicating that intracellular GSH depletion preceded cell toxicity.

Reactive oxygen species (H_2O_2) formation in human melanoma SK-MEL-28 cells

Reactive oxygen species (ROS) formation was investigated using the 2',7'-dichlorofluorescein assay 24 when APAP was incubated with human SK-MEL-28 cells. Our results indicate that APAP at concentrations ranging from 100-2000 μM at various times points (5-40 min after co-incubation) showed corresponding escalation in ROS formation (data not shown) in SK-MEL-28 cells. Additionally, co-incubation with CS (15 μM), an inhibitor of the PTP,27 caused a decrease in ROS formation in SK-MEL-28 cells from 17-fold by APAP (100 μM) to 13-fold when APAP (100 μM) was co-incubated with CS at 40 min (Fig. 6). Similar results were seen in the presence of TF (15 μM), a PTP inhibitor.27 Co-incubation of TF (15 μM) with APAP (100 μM) in SK-MEL-28 cells caused a decline in ROS formation at 40 min from 17-fold by APAP (100 μM) to 11-fold when APAP (100 μM) was co-incubated with TF (15 μM) (Fig. 6). Dicoumarol (10 μM), a diaphorase inhibitor,25 enhanced APAP (100 μM) induced ROS formation from 17-fold to 31-fold in SK-MEL-28 cells at 40 min incubation time (Fig. 6). 1-Bromoheptane (75 μM), a GSH depleting agent,26 increased APAP induced ROS formation in SK-MEL-28 cells from 17-fold to 36-fold (Fig. 6). The modulators caused no significant effect when incubated alone with SK-MEL-28 cells.

Computerized time-lapse microscopy is SK-MEL-28

Computerized time-lapse video microscopy confirmed that APAP treatment inhibits proliferation of SK-MEL-28 cells (Fig. 7). To quantify the inhibition of proliferation, cell division rates were calculated from a period when cells were growing for 24 h and then for another 48 h following exposure to either 100 μM, 200 μM APAP or DMSO (vehicle control). Statistical analysis of the time-lapse data was based on a linear mixed effects model in which there were fixed effect terms for a common slope of the division rate curve for both treatment groups during the first 24 h period (normal growth) and different slopes during the

second 48 h period when each group received different treatments. A random effect was added to account for differences among different microscopic fields within the cell culture plate. This analysis found that the slope of the curve for division rate/hour increased during the second 24 h period and the increase was significantly reduced for the group treated with APAP ($p<0.001$).Fig. 7 shows selected images from time-lapse videos highlighting the inhibition of cell division and induction of apoptotic-like cell death in cultures exposed to APAP. APAP at 100 μM demonstrated a significant anti-proliferative effect with significant less mitotic activity at 24 h and 48 h whereas at 200 μM APAP demonstrated cell toxicity at 24 h and no mitotic activity at 24 h and 48 h.

DISCUSSION

Treatment of metastatic melanoma by chemotherapeutic drugs is offset by problems of lack of specificity and toxicity.3 The relative resistance of malignant tumors to chemotherapy can be associated with higher GSH levels within these cells compared to normal tissues.29 APAP has been used effectively and safely by a large number of patients for its analgesic and antipyretic effects. GSH plays a key role in protecting cells from electrophilic compounds and free radicals generated during cellular metabolism. GSH is used by cells to detoxify reactive intermediates and is critical for elimination of many drugs, including APAP. Depletion of GSH can lead to tumor cell death *in-vitro*, especially in melanocytic cells that generate high levels of oxyradicals.11 It is also well known that N-acetyl cysteine is used as an antidote in acetaminophen induced toxicity mainly because it restores the GSH content in the liver.30, 31 In a previously reported study, Mitchell et al investigated GSH status to cellular integrity in cultured hepatocytes over time after initiation of chemical exposure. Their findings indicate that depletion of GSH to 20% of normal levels qualifies cells for significant drug/chemical induced injury.30 implying that that GSH depletion precedes cell toxicity. At its regular dose in the body, APAP is mainly detoxified either by glucuronide conjugation or sulfation.10 At overdose, it is also metabolized by CYP2E1 to form a reactive intermediate N-acetyl-para-benzoquinoneimine (NAPQI) that depletes GSH. Saturation of the detoxification pathways (conjugation and sulfation) causes' excess of NAPQI to be formed consequently leading to extensive depletion of GSH and thereby cell death.10

In addition, studies have shown that the cytotoxic antitumor activity of the quinone group is due to its ability to generate free radicals and active oxygen species through oxidation-reduction reactions. Free radicals generated may cause damage to critical targets macromolecules like DNA, RNA, and enzymes as well as structural components, such as proteins and lipids. 32, 33 In another study the mechanism of cytotoxicity of quinone antitumor agents was studied in L5178Y lymphocytes. It was shown that the cytotoxicity of quinone compounds was significantly inhibited by catalase, but was not affected by superoxide dismutase..34

In this study, we have tested APAP in nine cancer cell lines, five of which were melanoma cell lines which express functional tyrosinase, and four non-melanoma cell lines demonstrating that APAP was selectively toxic towards melanoma cell lines expressing tyrosinase (Fig. 5). The IC_{50} (48 h) for APAP in SK-MEL-28, MeWo, and SK-MEL-5 human melanoma cells was 100 μM which is within the APAP therapeutic concentration with an average peak concentration 132 μM (range: 66-200 μM)10 as an analgesic/ antipyretic/anti-inflammatory agent. We hypothesized that the biochemical mechanism of APAP toxicity in human melanoma cells is due to depletion of the cells' intracellular GSH stores ultimately leading to desirable and selective melanoma cell death. *In-vitro* enzyme assays performed using tyrosinase enzyme, a melanoma molecular target and CYP 2E1 induced rat liver microsomes showed APAP was metabolized significantly by tyrosinase

enzyme to give o-quinone while it was metabolized less by the CYP 2E1 induced rat liver microsomal preparation. Tyrosinase has previously been used as a molecular target in melanoma directed enzyme prodrug therapy.16, 28, 35 It has also been shown that tyrosinase expression in transfected nonmelanotic cells can be used to activate prodrugs for nonmelanotic cell treatment. The transfected cells were able to cause cell death attributable to prodrug conversion to a toxic product.36 APAP thus fulfils the role of a prodrug and is selectively bioactivated by melanoma tyrosinase to active quinone metabolites which are cytotoxic. The selective melanocytotoxicity of phenolic compounds has been related to the generation of radical species.37

In addition, we investigated the biochemical basis of APAP induced toxicity in human SK-MEL-28 melanoma cells using a number of modulators to enhance and/or prevent APAP toxicity. Our findings indicate that APAP toxicity towards SK-MEL-28 human melanoma cells was significantly enhanced by dicoumarol, a diaphorase inhibitor,25 and 1-bromoheptane, a GSH depleting agent,26 while ascorbic acid (AA), a reducing agent,9 and GSH precluded APAP toxicity, significantly. In order to investigate the mechanism of APAP induced toxicity in human SK-MEL-28 cells, the enzymatic oxidation of APAP by tyrosinase enzyme was followed using UV-VIS spectroscopy. In the absence of GSH a peak at 275-380 nm was developed. This did not develop when GSH was added prior to tyrosinase addition suggesting possible GSH conjugate formation with o-quinone. AA and NADH initially prevented the formation of 275-380 nm peaks, however, eventually the peak was formed after oxidation of AA and NADH as a result of the enzymatic oxidation of APAP mediated by tyrosinase. Our findings indicate that GSH was more effective in preventing AA and NADH depletion in tyrosinase/O_2/APAP bioactivation reaction. Ascorbic acid (AA), NADH and GSH are intracellular anti-oxidants. In addition, NADH plays a critical role as a cellular energy supply.9 The events related to AA, NADH, and GSH depletion and oxidation, and o-quinone formation and GS-conjugate formation are illustrated in Fig. 8. A similar mechanism of toxicity was described previously for 4-HA and ethyl 4-hydroxybenzoate induced toxicity in murine B16-F0 9 and SK-MEL-28 melanoma cells,35 respectively. Earlier, Valero et al studied the oxidation pathway of APAP12¯14 to its corresponding o-quinone by tyrosinase. In addition, the identification of a mono-glutathione conjugate for 4-methoxycatechol provided a strong evidence that 4-HA was metabolized by tyrosinase metabolizing system to o-quinone.

Malignant tumors, in comparison with normal tissues, are resistant to chemotherapeutic drugs. The resistance in most cases is associated with higher GSH levels within these cancer cells. Melanogenic cells use GSH as an intermediate in phaeomelanosynthesis,38 as well as for non-specific protection against cytotoxic intermediates of melanization. 39 Overexpression of GSH and GSH-dependent enzymes in melanotic melanoma cells could, therefore, be interpreted as an adaptive mechanism of these cells to oxidative stress resulting from melanogenesis. 40 It has also been reported that tyrosinase is present in relatively high concentrations in human melanoma tissue. 41 In a study of tyrosinase prepared from normal skin and metastatic melanoma from the same patient, 46 to 95 units per mg were found in the skin and 17,000 to 19,500 units per mg in the melanoma. 41 A decrease in the cellular levels of GSH brought about by a tyrosinase mediated prodrug bioactivation approach would therefore be more intense in melanoma cells in comparison to normal melanocytes. Thus, approaches to cancer treatment could potentially benefit from a selective GSH-depleting strategy in melanoma cells in comparison to normal melanocytes (Fig. 3). We studied the implications of intracellular GSH depletion on APAP cytotoxicity in human SK-MEL-28 cells. Determination of intracellular GSH was based on a modified GSSG reductase recycling method using a new reagent.23, 35 The method modified for measurement of intracellular GSH. In this measurement, GSH reacted with DTNB to form the disulfide GS-TNB and the yellow colored compound 5-thio-nitrobenzoic acid (TNB) was measured. The

disulfide products, GSSG (oxidized GSH) and GS-TNB, were subsequently reduced by GSSG reductase in the presence of NADPH, recycling GSH back to the reaction.35 Our data indicates that APAP caused a significant depletion of intracellular GSH after a short incubation time of 1-2 h without a significant change in cell viability thereby concluding that GSH depletion preceded cell toxicity.

Moreover, our studies into APAP induced ROS formation in SK-MEL-28 melanoma cells using the 2',7'-dichlorofluorescein assay 24 suggest that ROS formation plays a major role in APAP induced cell toxicity. CS and TF, inhibitors of the PTP in mitochondria,27 caused a decline in ROS formation, while BH, a GSH depleting agent,26 and DC, a diaphorase inhibitor,25 caused a significant increase in ROS formation when co-incubated with APAP in SK-MEL-28 melanoma cells. Our studies with CS, an inhibitor of permeability transition pore (PTP) protein in mitochondria,27 showed CS was significantly potent in inhibiting APAP toxicity towards melanoma cells. TF was equally effective in inhibiting PTP opening in melanoma cells thus limiting APAP mediated cell toxicity.

The Log P value for APAP was estimated to be 0.92 using LogP software, which indicates that APAP can readily partition in lipid phase and that APAP can readily pass through cell membranes and enter melanoma cells which can then be metabolized by tyrosinase to a catechol and then an o-quinone.

APAP has been shown to actively deplete intracellular stores of GSH, thus making it vulnerable to drug mediated cell toxicity and leading to desirable melanoma cell death. The dose of APAP used throughout this study was well within the therapeutic range currently being used for its analgesic antipyretic effect. Our study thus suggests that APAP was selectively toxic towards human SK-MEL-28, MeWo,SK-MEL-5, B16-F0, and B16-F10 melanoma cell lines when compared to non-melanoma BJ, Saos-2, SW-620 and PC-3 cells. Our data suggests that the formation of reactive species by tyrosinase is responsible for this selective toxicity. We therefore propose that APAP at its therapeutic concentration may be a suitable candidate for anti-melanoma therapy. Further investigation into APAP *in-vivo* toxicity and efficacy in melanoma tumor bearing mice is currently under investigation in our laboratory.

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Abbreviations list

APAP	Acetaminophen
ROS	Reactive oxygen species
MTT	(3-(4, 5-Dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide)
DETAPAC	Diethylenetriaminepentaacetic acid
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
AA	Ascorbic acid
BH	1-Bromoheptane

DC	Dicoumarol
TF	Trifluoperazine
CS	Cyclosporin
PTP	Permeability transition pore

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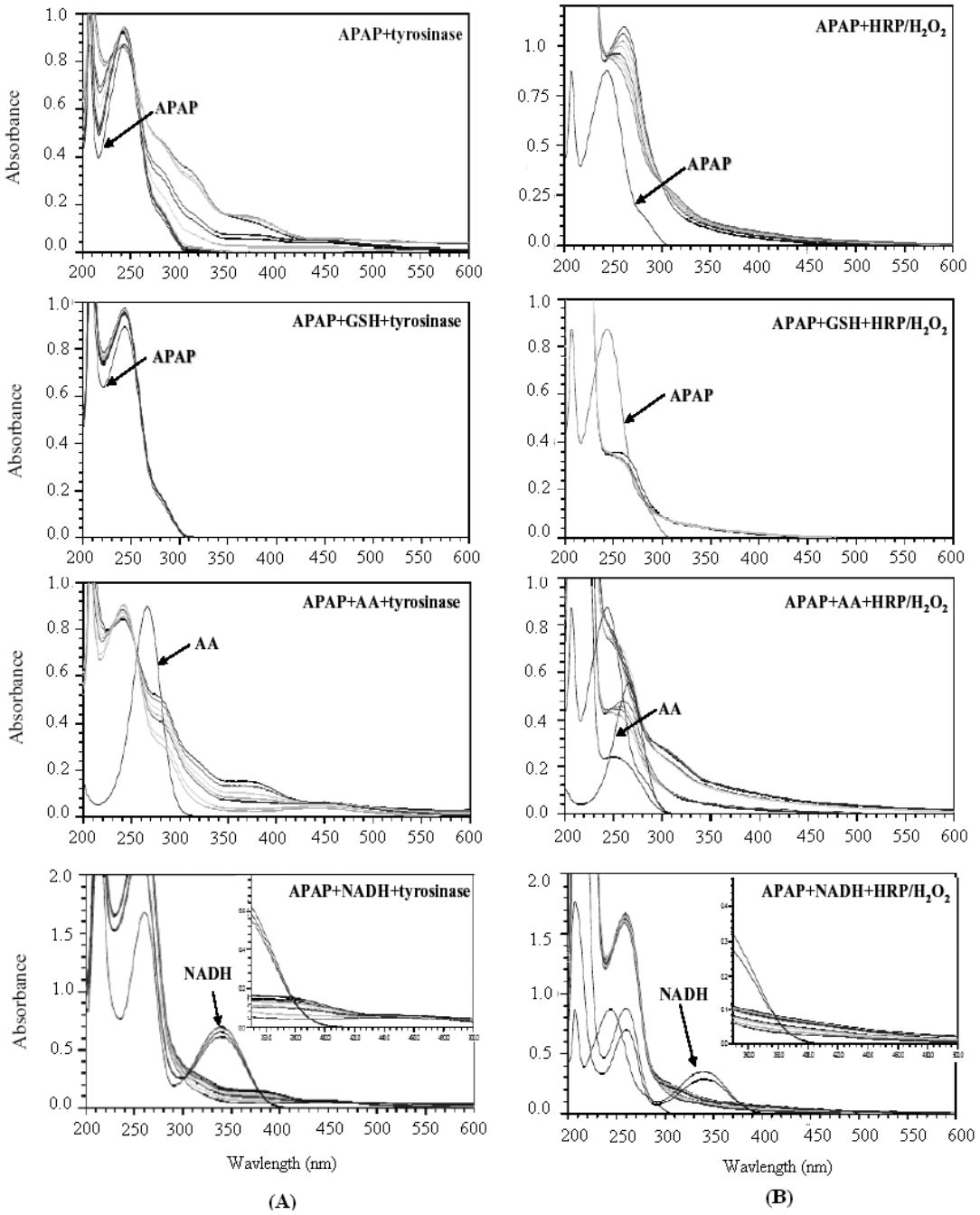

Figure 1.

UV-Vis overlay scan for the enzymatic oxidation of APAP by **(A)** tyrosinase/O_2 and **(B)** HRP/H_2O_2 at pH 7.4. Upon addition of tyrosinase enzyme to the APAP solution, an o-quinone peak developed at 275-380 nm. Addition of GSH prior to tyrosinase prevented the o-quinone peak formation at 275-380 nm. Addition of AA did not prevent formation of an o-quinone peak at 275-380 nm. The addition of NADH did not prevent the formation of the o-quinone peak at 380 nm. Addition of GSH, AA, and NADH did not prevent the polymerization and enzymatic oxidation of APAP by HRP/H_2O_2. The UV-VIS spectra were taken at 10 min intervals.

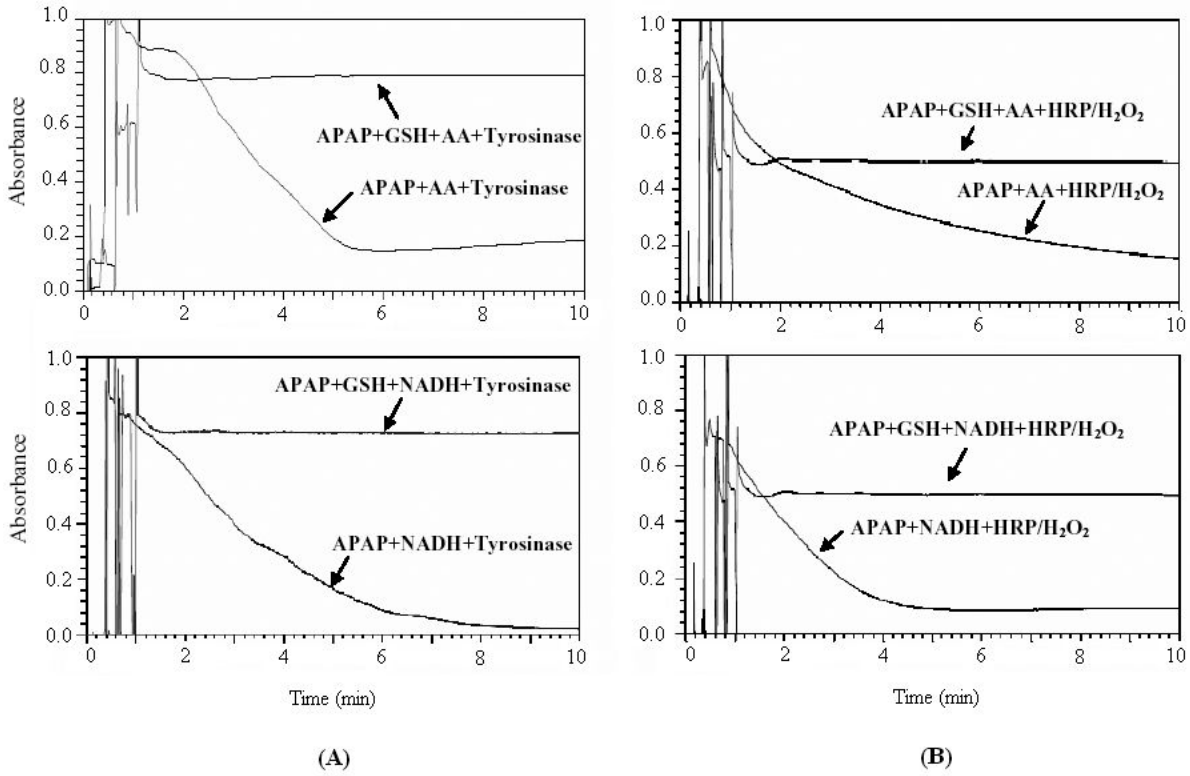

Figure 2.

Kinetic scan for the enzymatic oxidation of APAP by tyrosinase/O_2 and HRP/H_2O_2. AA and NADH oxidations were monitored at 266 nm and 340 nm, respectively. **(A)** The rate and extent of AA and NADH oxidation as a result of the enzymatic oxidation of APAP by tyrosinase/O_2 and **(B)** The rate and extent of AA and NADH oxidation as a result of the enzymatic oxidation APAP by HRP/H_2O_2 at pH 7.4. Addition of GSH prior to tyrosinase completely prevented AA and NADH oxidation. GSH diminished the rate of AA and NADH oxidations by APAP/ HRP/H_2O_2 metabolizing system.

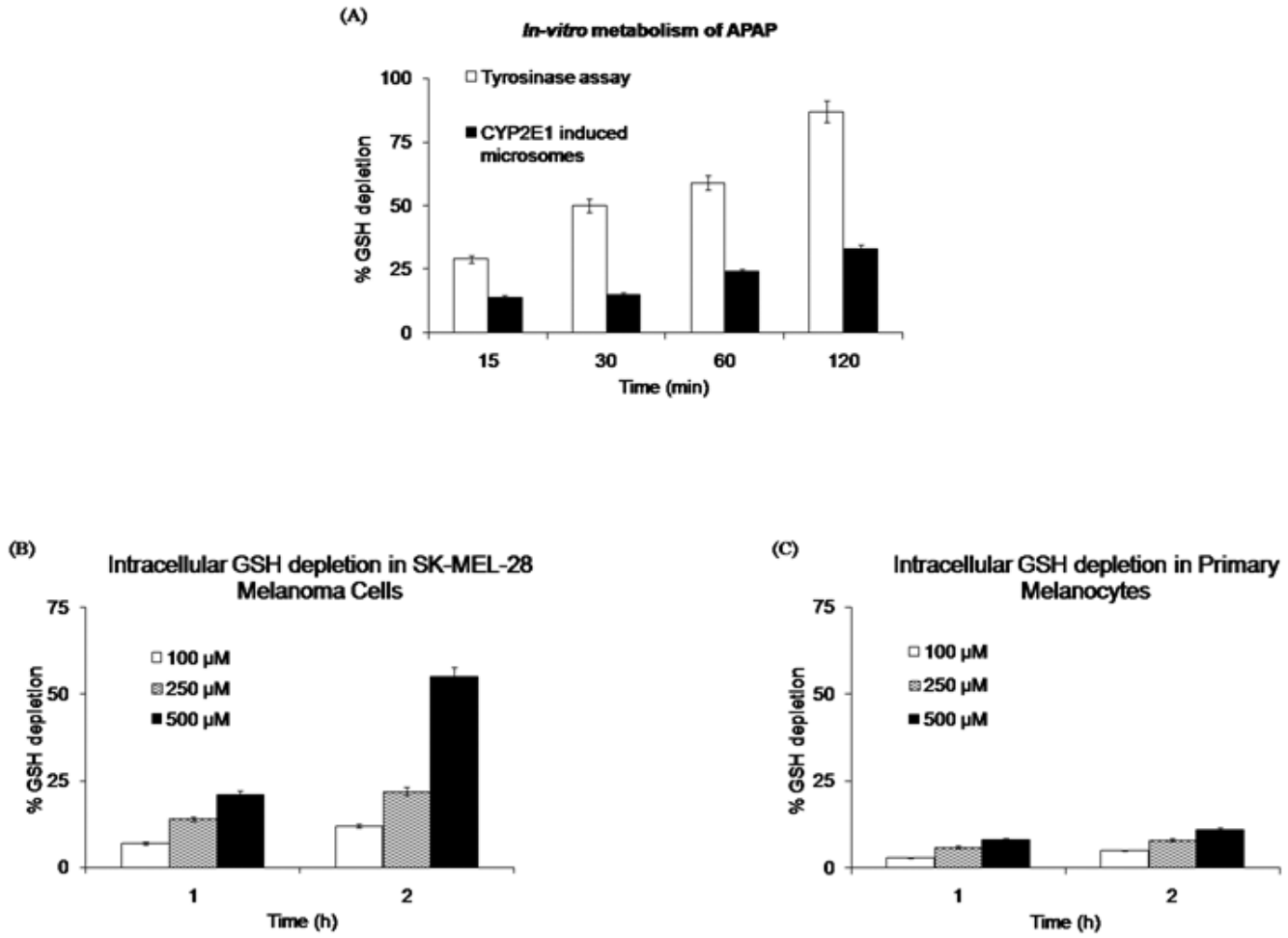

Figure 3.

(A) % GSH depletion mediated by the enzymatic oxidation of APAP by tyrosinase/O_2 and CYP2E1 induced rat liver microsomes. On a molar basis, 1.8 and 0.6 mol GSH was depleted when APAP was enzymatically oxidized by tyrosinase/O_2 and CYP2E1 induced rat liver microsomes, respectively. **(B)** Intracellular GSH depletion by (100, 250, 500 µM) in SK-MEL-28 cells (1, and 2 h). **(C)** Intracellular GSH depletion by (100, 250, 500 µM) in Human Epidermal Melanocytes (normal cells) (1, and 2 h).

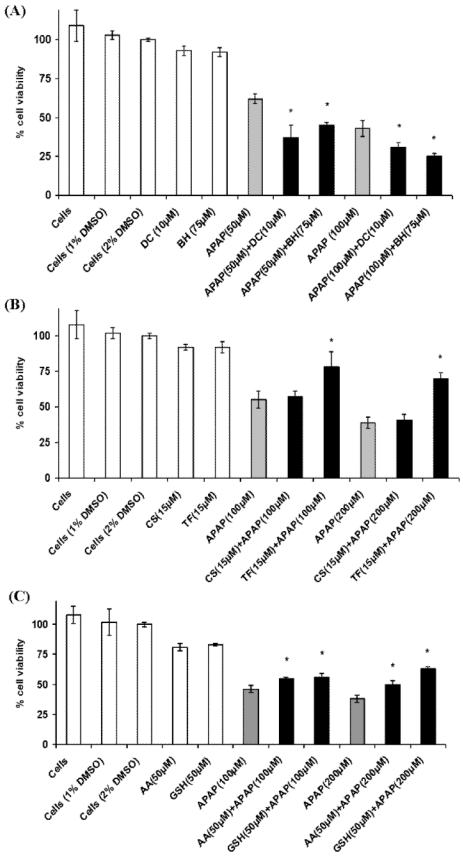

Figure 4.

(A) Toxicity of APAP in the presence of dicoumarol (DC) and 1-bromoheptane (BH) (48 h); DC, a diaphorase inhibitor, and BH, an intracellular GSH depleting agent significantly increased APAP (100 μM) toxicity in SK-MEL-28 melanoma cells. **(B)** Toxicity of APAP in the presence of Cyclosporin A (CS) and Trifluoperazine (TF) (48 h); TF, an inhibitor of PTP in mitochondria reduced APAP toxicity in SK-MEL-28 cells. **(C)** Toxicity of APAP in the presence of Ascorbic acid (AA) and GSH (48 h); AA, a reducing agent and GSH significantly reduced APAP toxicity (*significantly different).

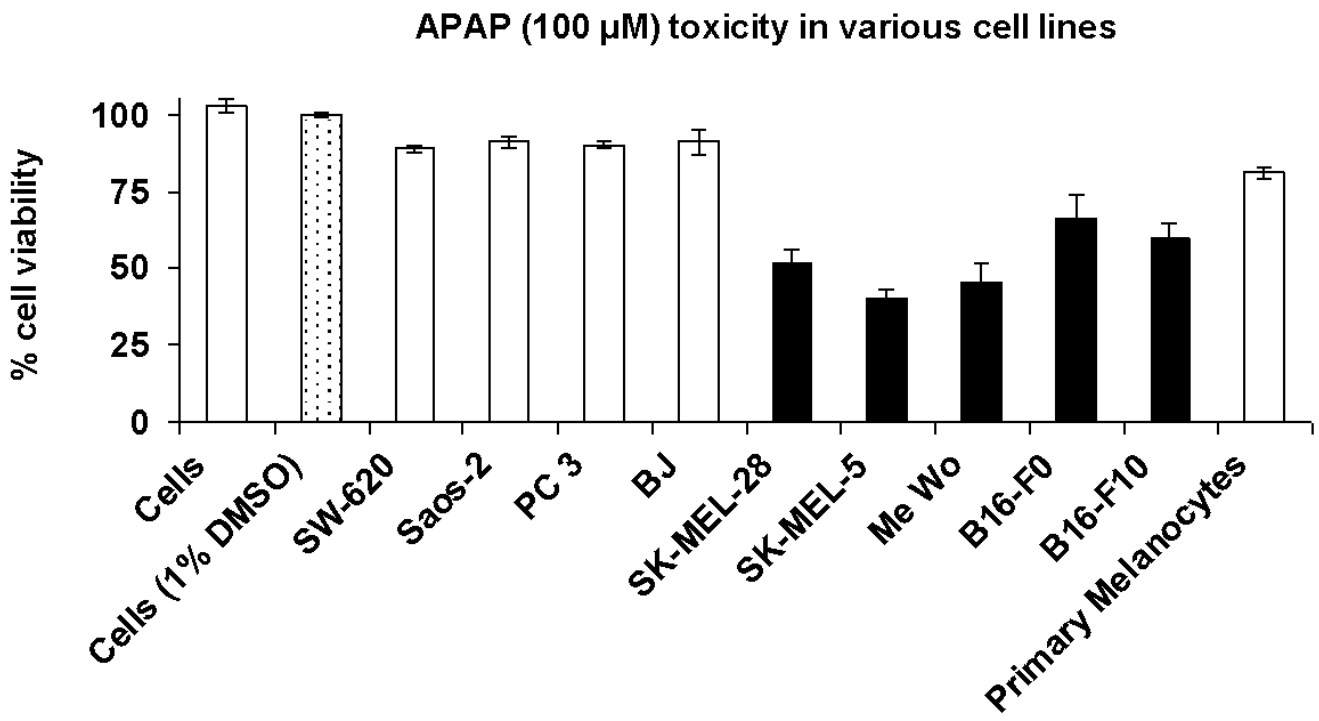

Figure 5.

APAP toxicity in non-melanoma and melanoma cell lines. APAP (100 μM) demonstrated negligible toxicity in BJ skin fibroblast, prostate (PC-3), colorectal adenocarcinoma (SW-620) and osteosarcoma (Saos-2)cells; while it showed considerable toxicity in metastatic melanoma cell lines (SK-MEL-28, SK-MEL-5, MeWo, B16-F0 and B16-F10) at 48 h incubation time demonstrating that APAP was selectively toxic towards melanoma cells but not against non melanoma cell lines. It showed moderate toxicity against primary normal melanocytes.

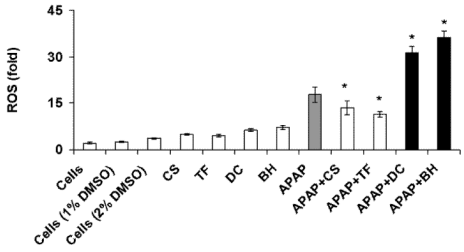

Figure 6.

ROS (fold) formation (at 40 min) in the presence of modulators in SK-MEL-28 cells. Co-incubation of CS (15 μM) and TF (15 μM), inhibitors of the PTP in mitochondria with APAP (100 μM) caused a significant decline in ROS (fold) formation in SKMEL-28 melanoma cells. Co-incubation of APAP (100 μM) with DC (10 μM), a diaphorase inhibitor and BH, an intracellular GSH depleting agent showed a corresponding escalation in ROS (fold) formation in SK-MEL-28 melanoma cells. (*significantly different). 1-Bromoheptane (BH); Dicoumarol (DC); Trifluoperazine (TF); Cyclosporin A (CS),

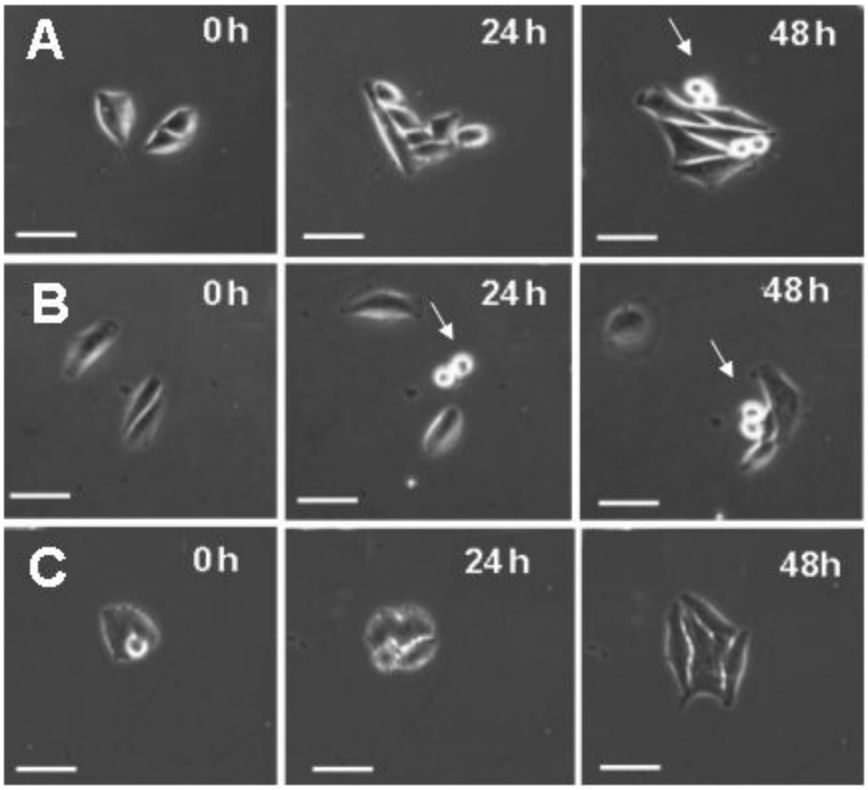

Figure 7.

Phase contrast images from time-lapse videos of SK-MEL-28 cells treated with APAP at A) 0 μM, B) 100 μM and C) 200 μM. Elapsed time in hours is indicated in upper right corner of each panel with the start of drug treatment designated at time equals 0 h. No cells entered the microscopic field after the initial frame. Bar = 60 microns. Arrow indicates cells undergoing mitotic division.

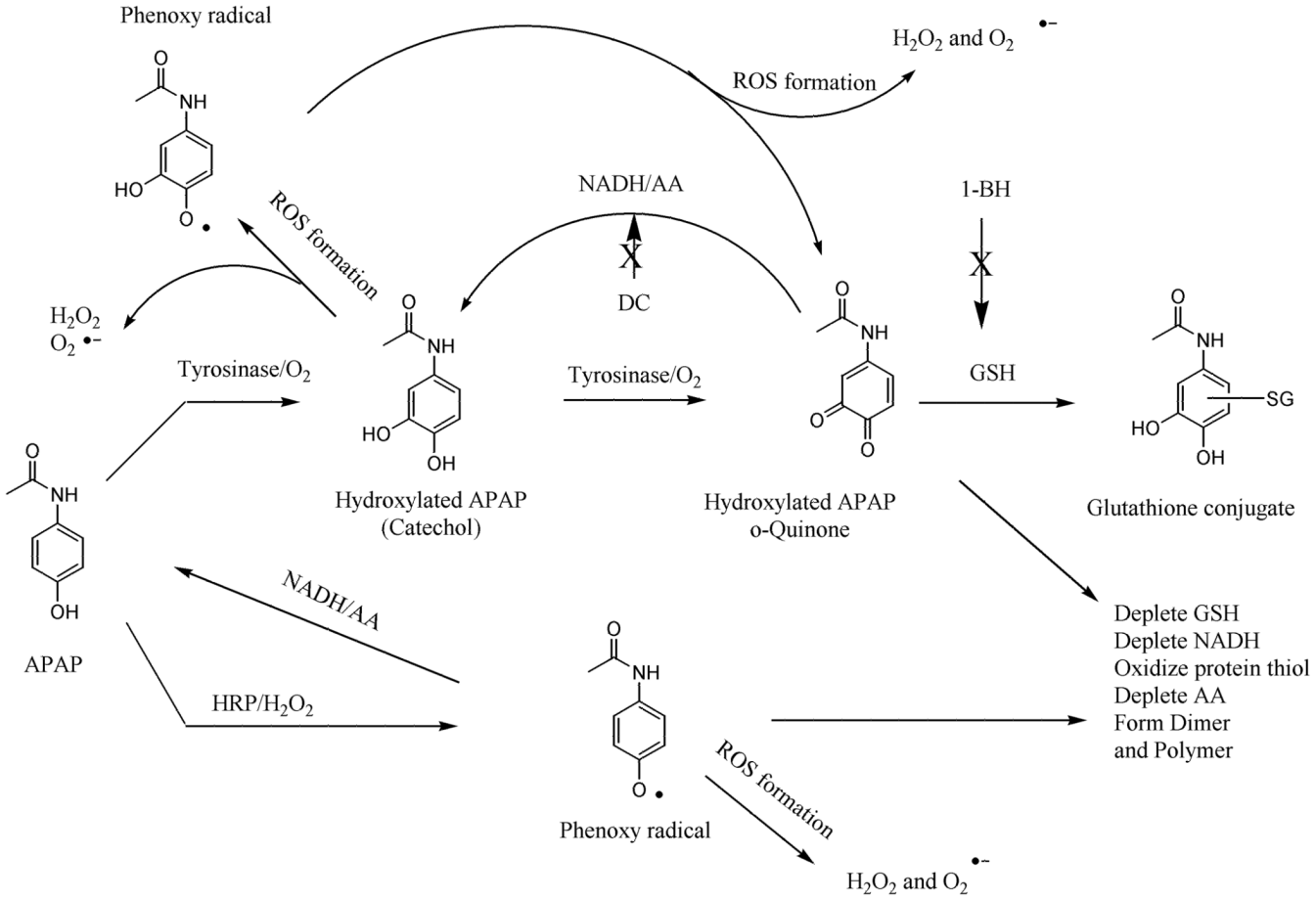

Figure 8.

Proposed biochemical mechanism for APAP toxicity in SK-MEL-28 melanoma cells. AA, NADH and GSH are intracellular anti-oxidants. In addition NADH plays a critical role as a cellular energy supply. APAP toxicity towards SK-MEL-28 human melanoma cells was significantly enhanced by dicoumarol (DC), a diaphorase inhibitor, and 1-bromoheptane (BH), a GSH depleting agent, while ascorbic acid, a reducing agent and GSH precluded APAP toxicity, significantly.