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Original article

Development of novel naphthalimide derivatives and their evaluation as potential melanoma therapeutics

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ABSTRACT

Synthesis and anti-melanoma activity of various naphthalimide analogs, rationally modified by introducing isothiocyanate (ITC) and thiourea (TU) functionalities, found in well-known anti-cancer agents, is described. The structure–activity relationship comparison of the novel agents in inhibiting cancer cell growth was evaluated in various melanoma cell lines. Both ITC and TU analogs effectively inhibited cell viability and induced apoptosis in various human melanoma cells. Nitro substitution and increase in alkyl chain length, in general, enhanced the apoptotic activity of ITC derivatives. All the new compounds were well tolerated when injected intraperitoneal (i.p.) in mice at effective doses at which both the ITC and TU derivatives inhibited melanoma tumor growth in mice following i.p. xenograft. The nitro substituted naphthalimide–ITC derivative **3d** was found to be the most effective in inducing apoptosis, and in inhibiting melanoma cell and tumor growth.

1. Introduction

Over the years, several naphthalimide analogs with varying efficacy have been reported as having promising anti-cancer properties [1–6]. Studies have led to the identification of some potent analogs such as mitonafide and amonafide, which have shown an interesting anti-tumor activity both preclinically [7] and in phase I and phase II clinical trials [8–10]. However, the trials were associated with severe toxicity issues [8–11]. These observations warranted structural optimization by keeping the key naphthalimide moiety intact while adding appropriate functional groups that could help reduce systemic toxicity.

Herein, we made use of isothiocyanate (ITC) and thiourea (TU) functionalities to replace the *N*,*N*-dimethyl group (Fig. 1). The rationale for introducing ITC functionality was that naturally occurring ITC compounds, that are stored as glucosinolates, in plants and cruciferous vegetables such as watercress, broccoli, cabbage, cauliflower etc., have been well-known for their anti-cancer properties [12–14]. Numerous epidemiological studies have reported lower cancer risk among individuals who consume large quantities of cruciferous vegetables, which have shown to prevent cancer at a variety of organ sites [15,16]. The ITCs are shown to be effective anti-cancer agents both at initiation [17–22] (inhibiting Phase I and inducing Phase II enzymes) and post initiation and progression [21–25] (block cell-cycle progression and induce apoptosis in human cancer cells) stages of several cancers. ITCs have also been shown to suppress metastasis via inhibition of invasion and migration of human hepatoma cells [26,27], and inhibit prostate cancer progression and pulmonary metastasis by reducing cell proliferation and augmenting natural killer cell lytic activity [28]. Furthermore, our recent reports have shown ITCs to be effective in inhibiting melanoma tumor growth without any systemic toxicity issues [29,30]. Therefore, the associated dual anti-cancer properties i.e. being capable inhibiting both tumor growth and metastasis, favorable mechanism of action, and safety profile associated with ITC (–N=C=S) functionality prompted us to incorporate this group into naphthalimide moiety for structural optimization.

Furthermore, we also synthesized a series of naphthalimide analogs by incorporating a thiourea functionality in place of *N*,*N*-

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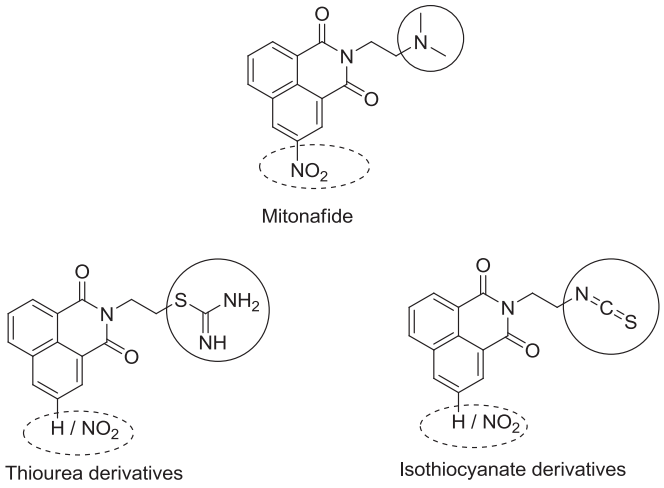


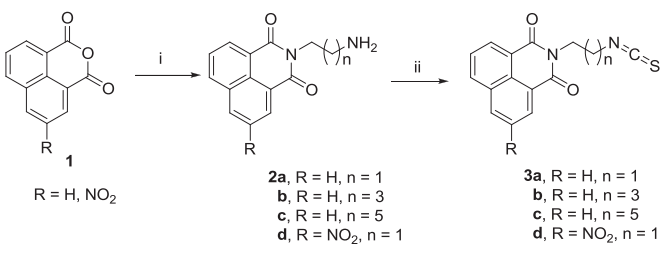
Fig. 1. Structure of mitonafide and general structures of novel naphthalimide derivatives.

dimethyl in mitonafide, with or without nitro substitution (Fig. 1). Thiourea functionality is a part of well-known chemopreventive agents such as *S*,*S*′-1,4-phenylenebis(1,2-ethanediyl)bis-isothiourea (PBIT) and S-methylthiourea. These agents are known to exert their chemopreventive efficacy by inhibiting inducible nitric oxide synthase (iNOS), a calcium-independent, cytokine-inducible enzyme involved in the production of a bioactive, pleiotropic regulatory and signaling molecule from arginine called nitric oxide [31]. Recent studies have implicated iNOS as a potential therapeutic target in melanoma [32–36]. Therefore, we synthesized naphthalimide thiourea (NTU) derivatives to test on in vitro and in vivo melanoma models. In brief, we describe herein, synthesis of various aforementioned rationally modified naphthalimide derivatives with a variety of functional alterations and evaluation of their effectiveness to inhibit cell viability and induce apoptosis in human melanoma cells, and for inhibiting tumor growth in xenograft melanoma models.

2. Results

2.1. Chemistry

The synthetic route to obtain the naphthalimide–ITC derivatives (**3a**–**d**) is depicted in Scheme 1. The key precursors in the synthesis i.e. the aminoalkyl-naphthalimides **2a**–**d**, were synthesized by refluxing 1,8-naphthalic anhydride or 3-nitro-1,8-naphthalic anhydride with appropriate diaminoalkane as reported in the literature [37,38]. The compounds **2a**–**d** were converted to the desired ITC-derivatives **3a**–**d**, with a little modification of the literature method [39], by reacting with thiophosgene in chloroform in the presence of potassium carbonate. The use of potassium carbonate as a base accelerated the reaction.



Scheme 1. Synthesis of naphthalimide-isothiocyanate derivatives. Reagents and conditions: (i) diaminoalkane, EtOH, reflux, 2 h (ii) thiophosgene, K_2CO_3, $CHCl_3$, RT, 2 h.

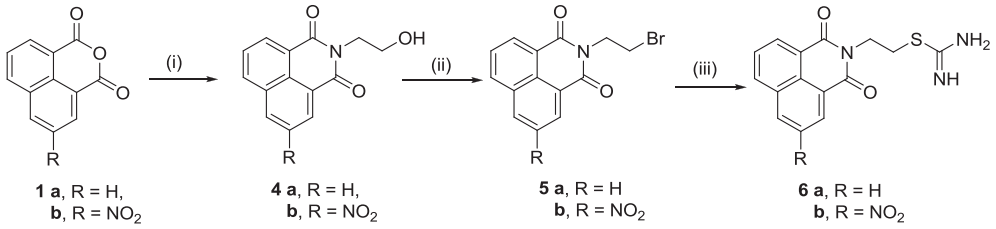
The thiourea derivatives were synthesized according to Scheme 2. Compounds **4a** and **5a** were synthesized as reported previously [40] and were characterized by comparing with the literature data. The nitro substituted analogs **4b** and **5b** were obtained following a similar methodology. Refluxing 1,8-naphthalic anhydride/or 3-nitro-1,8-naphthalic anhydride with ethanolamine in ethanol gave alcohols **4a**,**b** which on treatment with PBr_3 in refluxing ethyl acetate yielded the key intermediate bromoalkyl naphthalimides **5a**,**b**. The desired compounds **6a** and **6b** were finally obtained by refluxing **5a**,**b** with thiourea in acetonitrile. All the compounds were purified by silica gel column chromatography and characterized on the basis of ^{1}H NMR and high-resolution MS.

2.2. Naphthalimide analogs induce human melanoma cancer cell cytotoxicity

A colorimetric cell proliferation assay was used in multiple human melanoma cell lines such as UACC 903, A375-S2, SK-MEL-37 and 1205 Lu to assess the effect on cell proliferation of novel naphthalimide derivatives. Melanoma cells (5×10^3) were plated and grown for 24 h in 96-well plates, and then treated for 24 or 72 h with various concentrations (0.5, 1, 5, 10, 25 and 50 μM in DMSO) of each compound or control DMSO. After the incubation period, the number of viable cells was determined by measuring the bioreduction by intracellular dehydrogenases of the tetrazolium [41] compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) in the presence of the electron coupling reagent PMS (phenazine methosulfate). The assay showed a dose-dependent decrease in cell viability for all the compounds (Fig. 2). Both the ITC and TU naphthalimide derivatives with two carbon alkyl chain, **3a** and **6a**, exhibited comparable cytotoxicity in UACC 903, A375-S2, and SK-MEL-37 cell lines, while in 1205 Lu cells, **3a** was more effective. Nitro substitution enhanced the cytotoxicity in case of ITCs; 3-nitro substituted naphthalimide–ITC derivative with two carbon alkyl chain length (**3d**) showed consistently low IC_{50} values in all the four cell lines as compared to the corresponding unsubstituted **3a** (Table 1). On the other hand, nitro substitution led to decrease in cytotoxicity in case of TU derivatives; nitro substituted **6b** was actually less effective than **6a**. There was a trend of increase in cytotoxicity with increase in alkyl chain length from 2 to 6 of ITC analogs in most cases. Compound **3d** was found to be most cytotoxic among the novel agents.

2.3. Naphthalimide analogs induce apoptosis in human melanoma cancer cells

To evaluate the apoptotic potency of novel naphthalimide derivatives and quantify the effect of increasing alkyl chain length and nitro substitution on these agents, apoptosis assay was carried out in UACC 903 human melanoma cells. Apoptotic cells were determined by the 7-Aminoactinomycin D (7-AAD) method [42–44], and apoptosis was measured using the PE Annexin V positive cells and 7-AAD staining cells were analyzed and quantified by using flow cytometry (BD FACScan). All these studies were conducted in three separate experiments. The cells were incubated at 37 °C without or with different concentrations (1, 5, and 25 μM) of ITC and TU derivatives for 24 h. At the end of incubation, treated and untreated cells were harvested by low speed centrifugation, stained with Annexin and 7-AAD and analyzed by flow cytometry to determine extent of apoptosis (Fig. 3). The results exhibited that the number of apoptotic cells increased with increasing alkyl chain length of ITC derivatives from 2 to 6 carbons (**3c** > **3b** > **3a**).



Scheme 2. Synthesis of naphthalimide-thiourea derivatives. Reagents and conditions: (i) Ethanolamine, EtOH, reflux, 1 h (ii)PBr_3, EtOAc, 80 °C, 2 h (iii) thiourea, CH_3CN, reflux, 4 h.

Interestingly, the incorporation of nitro substitution at 3-position of NITC-2 enhanced apoptosis, the effect was especially prominent at higher concentration (25 μM). On the other hand, the nitro substitution at 3-position of **6a** did not show any change on apoptosis induction; **6b** was in fact slightly less effective than **6a**.

2.4. Determination of maximum tolerated dose (MTD) of novel naphthalimide analogs

To assess the systemic toxicity level of new naphthalimide analogs, we carried out a maximum tolerated dose (MTD) study of these compounds. The Swiss Webster mice ($n = 4$) were treated i.p. every day with varying doses (5, 7.5, 10, and 15 mg/kg body weight) of the novel naphthalimide derivatives for 28 days. The body weight of animals was noted twice per week. The results indicated that all the mice treated with up to 10 mg/kg body weight dose of novel derivatives tolerated the drugs well as signified by lack weight loss or deaths. Only at a dose of 15 mg/kg, a few mice were dead in treatment groups.

2.5. Inhibition of melanoma tumor growth using novel naphthalimide analogs

After demonstrating that the naphthalimide derivatives were effective in killing various cancer cells, induced apoptosis in melanoma cells, and were well tolerable at a daily doses up to 10 mg/kg when administered i.p. to mice, we next examined if these new compounds were effective in inhibiting tumor growth at tolerable doses. Though none of the mice died at a dose of 10 mg/kg dose, the nitro substituted derivatives **3d** and **6b** treated mice showed reduction in body weight by about 10% by the end of the treatment period (28 days daily injection) in MTD studies. To stay

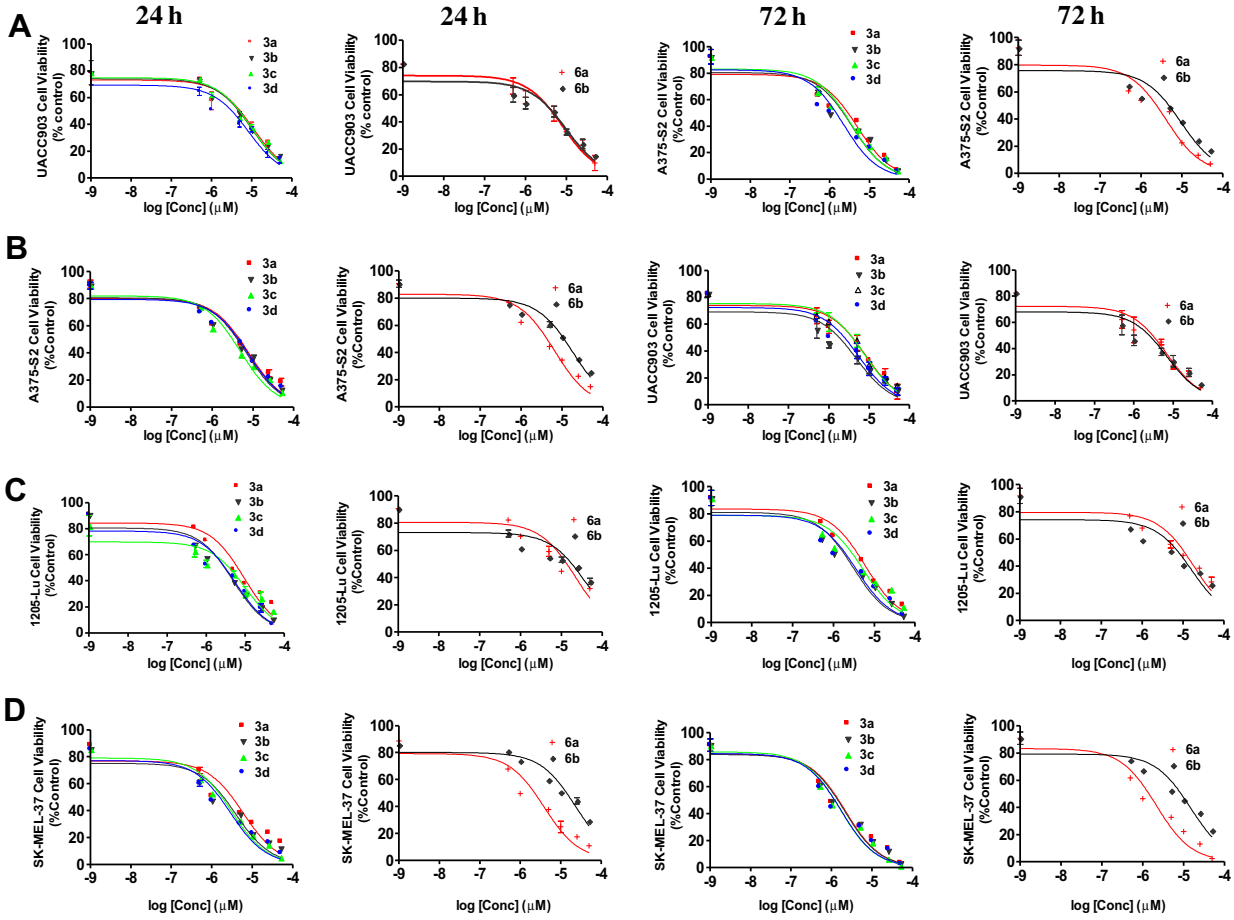


Fig. 2. Dose-response curves representing naphthalimide derivatives induced cell death in (A) UACC 903, (B) A375-S2, (C) 1205 Lu, and (D) SK-MEL-37 cells using the MTS assay. Cells were incubated with naphthalimide derivatives ranging from 0 to 50 μM (0.5, 1, 5, 10, 25 and 50 μM) at 37 °C for 24 and 72 h. The control cells were treated with DMSO. The product formazan was measured at 492 nm. The results were the mean of 3 different readings within one experiment, and the figures were representative of three independent experiments. Survival was expressed as the percentage of viable cells in treated samples relative to untreated control cells.

Table 1
IC_{50} (μM) of novel naphthalimide derivatives (MTS assay[a]) on human melanoma cells (24 and 72 h treatment).

Compounds	Human melanoma cell lines IC_{50} (μM)							
	UACC 903 (24 h)	UACC 903 (72 h)	A375-S2 (24 h)	A375-S2 (72 h)	SK-MEL-37 (24 h)	SK-MEL-37 (72 h)	1205 Lu (24 h)	1205 Lu (72 h)
3a	11.32 ± 1.1	8.04 ± 0.5	7.93 ± 1.1	5.05 ± 0.5	5.93 ± 0.9	2.20 ± 0.4	9.41 ± 1.2	6.04 ± 1.2
3b	10.70 ± 2.1	5.17 ± 0.4	6.89 ± 0.9	3.47 ± 0.5	3.89 ± 0.8	2.12 ± 0.1	4.88 ± 0.3	3.03 ± 0.3
3c	9.02 ± 1.7	7.60 ± 0.8	5.07 ± 0.6	3.54 ± 0.2	3.07 ± 0.4	1.67 ± 0.1	11.48 ± 0.9	5.25 ± 0.2
3d	8.38 ± 1.9	5.50 ± 0.3	7.88 ± 0.8	2.81 ± 0.3	2.88 ± 0.7	1.73 ± 0.2	5.82 ± 1.2	3.91 ± 1.1
6a	9.87 ± 1.3	6.85 ± 1.0	6.70 ± 1.1	3.78 ± 0.1	3.70 ± 0.2	2.15 ± 0.7	22.35 ± 3.3	18.73 ± 1.3
6b	14.74 ± 2.0	6.95 ± 0.6	18.44 ± 2.4	9.48 ± 1.2	23.44 ± 2.5	14.95 ± 1.9	39.47 ± 3.5	16.37 ± 2.1

[a] All values represent the average from three independent experiments; Values are mean ± S.E.

well within the safety limits for tumor growth inhibition study, we used near 10 mg/kg dose only 3 days a week instead of daily injections. The efficacy of selected naphthalimide compounds for inhibiting tumor development at this dosing regimen was evaluated in preclinical mouse model of melanoma. Athymic nude mice were subcutaneously injected with UACC 903 cells and tumor development was allowed to occur for six days, by which time tumors have undergone vascularization (angiogenesis). Mice were then treated i.p with naphthalimide derivatives three times a week on Mondays, Wednesdays and Fridays. At a dose of 0.6 μmole (8.5–10 mg/kg), ITC derivatives showed a 30–60% reduction in tumor growth (Fig. 4A). Compound **3d** was most effective in inhibiting tumor development (~60%), while **3c** showed nearly 50% inhibition. Compounds **3a** and **3b** showed a similar inhibition of about 30%. Furthermore, though, **3a** and **3b** were comparable, **3c** exhibited better inhibition indicating that increase in chain length may enhance potency. The comparison of **3a** and **3d** clearly demonstrated the enhancement of potency with nitro substitution. Interestingly, among TU analogs, the nitro substituted **6b** was actually slightly less effective than **6a**, when administered i.p. at a dose of 0.5 μmole (9.5–10.6 mg/kg) (Fig. 4B). These results were consistent with those obtained from in vitro cell viability and apoptosis assays. No evidence of systemic toxicity was observed at the doses used for any of the naphthalimide derivatives as evidenced by the body weights (Fig. 4C and D).

3. Discussion

Melanoma is the most deadly form of skin cancer due to its high metastatic potential. However, no effective therapy is available for treatment once the cancer advances and metastasizes to other parts of the body. Dacarbazine, the only FDA-approved chemotherapy drug for metastatic melanoma, is unfortunately not very effective: a recent review of the research showed an average response rate of only 15.3% with no significant improvement in overall survival. We have earlier discovered phenylbutyl isoselenocyanate (ISC-4) as an effective agent inhibiting melanoma tumor growth by about 60% at MTD (0.76 μmole) [29,30]. During the course of this study, we recognized that the isothiocyanate functionality was capable of inhibiting melanoma tumor growth. We also showed that ITC functionality makes the agents capable of inhibiting the Akt pathway; Akt3 is elevated in 70% of sporadic melanomas. In our efforts to develop better drugs for melanoma, we identified the naphthalimide moiety, which has been a core structure of several anti-cancer agents such as mitonafide and amonafide etc., to be modified to work against melanoma by incorporating functionalities such as ITC or TU. Therefore, we developed several derivatives bearing naphthalimide moiety attached to ITC and TU functionalities as potential anti-cancer agents.

The activity of phenylalkyl-ITCs [45–48] has been shown to vary with varying alkyl chain length. While some reports show the increase in potency with increasing alkyl chain length [45–47], others have reported an opposite effect [48]. In the present case, the increasing alkyl chain length linking naphthalimide to ITC functionality showed a moderate increase in cytotoxicity of these molecules in MTS assays. However, there was a clear increase in apoptosis induction with increasing alkyl chain length linking naphthalimide moiety to ITC functionality. Also, in melanoma xenograft model, the six carbon alkyl chain containing compound **3c** was most effective; while **3a** and **3b** were comparable showing that increase in alkyl chain length may enhance the potency of these compounds. Therefore, overall, the increase in alkyl chain length from 2 to 6 carbons did help enhance the potency of ITC derivatives. The addition of a nitro group at 3-position also led to a significant increase in the efficacy of ITC derivatives. **3d** was more effective than **3a** in inhibiting cell growth, inducing apoptosis, and inhibiting tumor growth. On the other hand, the addition of nitro group actually led to reduction in the efficacy of TU analogs.

Compound **3d** emerged as the most effective of all the compounds studied here. It showed similar inhibition at lower dose (0.6 μmole) than our previously reported compound ISC-4 (0.76 μmole; MTD) [29,30] at a 3 days/week dosing regimen, but was more tolerable. Our MTD studies have revealed that it may be delivered daily at this dose daily without any weight loss or apparent systemic toxicity issues. Therefore **3d** possesses a higher therapeutic index and may be clinically more relevant.

The mechanistic pathways responsible for the drop in toxicity of the novel agents are still unknown. However, based on literature evidences [49–51], the compounds with –N=C=S functionality, that is a part of naturally occurring isothiocyanates, metabolize and eliminate from the body mainly through mercapturic acid pathway i.e. by formation of nonenzymatic and enzymatic conjugation with glutathione (GSH) to give thiol conjugates. These upon stepwise enzymatic hydrolysis, lead to L-cysteine conjugates and subsequently *N*-acetyl-L-cysteine conjugates upon acetylation, and excrete from the body. This detoxification pathway seems responsible for the lower toxicity of naphthalimide–ITC derivatives.

4. Conclusion

Several naphthalimide analogs bearing ITC and TU functionalities have been developed. Both, the ITC and TU analogs of naphthalimide derivatives exhibited efficient inhibition of melanoma cell growth, were effective in inducing apoptosis in melanoma cells and effectively inhibited melanoma tumor growth without any systemic toxicity. Furthermore, in general, the efficacy increased with increasing alkyl chain length in case of ITC compounds. In preclinical melanoma xenograft model, the ITC compounds in general showed better tumor inhibition as compared to TU derivatives without any measurable systemic toxicity. Tumor inhibitory effect increased with increasing alkyl chain length in ITCs and by introducing nitro group at 3-position of naphthalimide moiety. Collectively, based on both in vitro and in vivo experiments, compound **3d** was found to be the most effective agent among all the reported compounds, in inhibiting cell growth, inducing apoptosis and in inhibiting melanoma tumor growth in nude mice

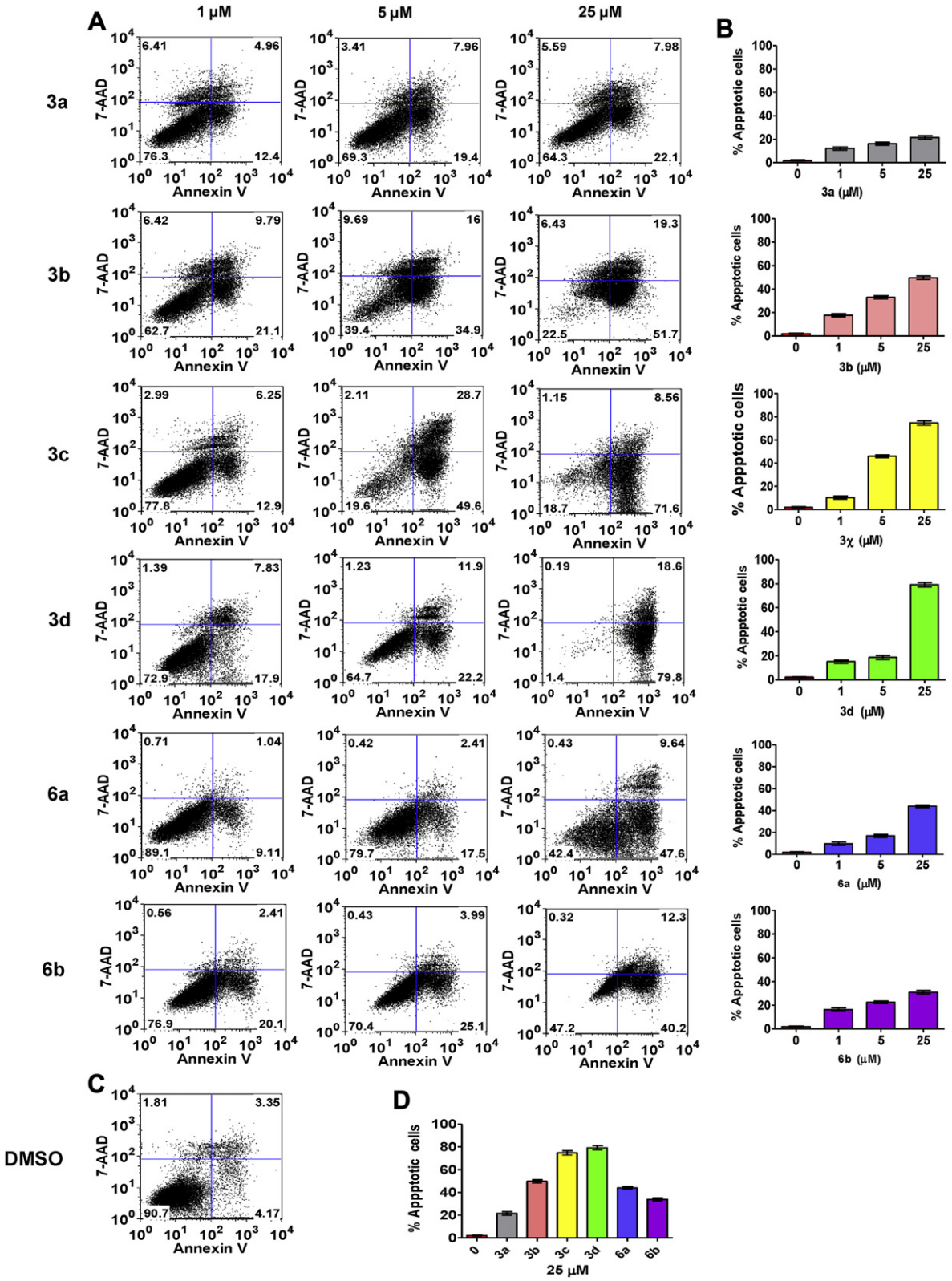


Fig. 3. Scattergrams of 7-AAD and PE Annexin V stained human melanoma UACC 903 cells are shown. (A) UACC 903 cells were treated with 0, 1, 5 and 25 μM naphthalimide derivatives such as **3a**, **3b**, **3c**, **3d**, **6a** and **6b** for 24 h at 37 °C. After incubation with 7-AAD and PE Annexin V the cells were analyzed by flow cytometry within 1 h; (B) The bar graphs representing a dose-dependent increase in % apoptotic cells on treatment with various naphthalimide derivatives. The results are expressed as percentage of Annexin V positive cells (early stage of apoptosis); (C) Control UACC 903 cells treated with DMSO; (D) Data are expressed as percentage of Annexin V positive cells at 25 μM concentration of each derivative. Increase in chain length and nitro substitution enhanced the apoptosis.

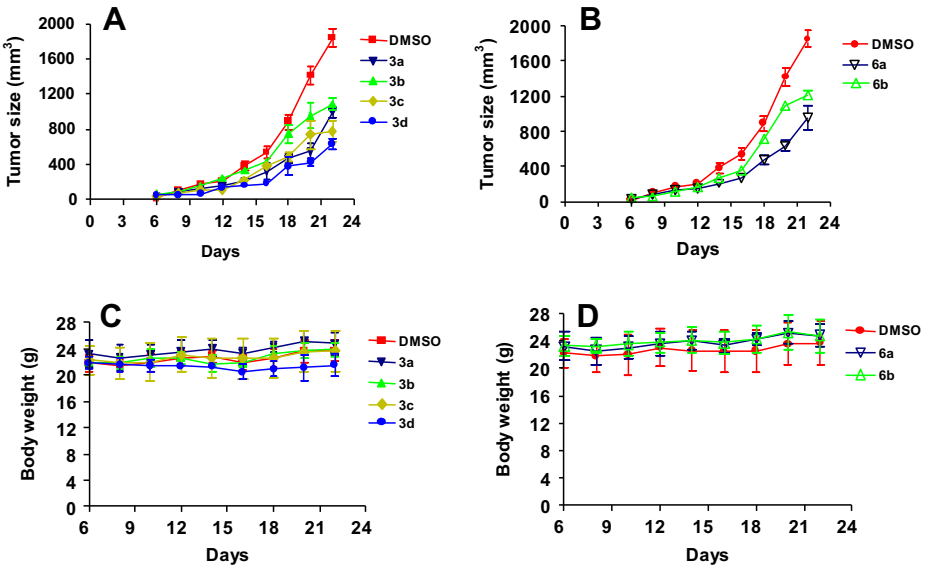


Fig. 4. In vivo melanoma tumor inhibition using novel naphthalimide derivatives. Six days after subcutaneous injection of UACC 903 melanoma cells, nude mice were treated i.p. with compounds (0.6 μmole for ITC and 0.5 μmole for TU derivatives) thrice per week or 0.6 μmole of 3a daily; (A) **3d** significantly reduced tumor growth (~60%) compared to DMSO control, while **3c** showed ~50% reduction. The **3a** and **3b** showed 30–35% inhibition in tumor growth. At daily dose 3a showed about 50% inhibition; (B) **6a** and **6b** showed about 20–30% reduction in tumor growth. The differences in the tumor growth rate between the treatment groups and the DMSO control groups were statistically significant ($^*P < 0.05$) according to two-way RM-ANOVA; (C,D) Body weights of treated mice compared to the control DMSO vehicle treated mice showed no significant differences between groups demonstrating negligible systemic toxicity.

without any systemic toxicity at effective dose; **3c** was only slightly less effective in all assays. These compounds thus hold solid promise of being effective therapeutic agents for melanoma.

5. Experimental section

5.1. Chemistry

Melting points were recorded on a Fischer–Johns melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance 500 MHz spectrometer. Chemical shifts (δ) were reported in parts per million downfield from the internal standard. The signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet), and dt (doublet of triplet). High-resolution MS (EI) were determined at the Chemistry Instrumentation Center, State University of New York at Buffalo, NY. Thin-layer chromatography (TLC) was developed on aluminum-supported pre-coated silica gel plates (EM industries, Gibbstown, NJ). Column chromatography was conducted on silica gel (60–200 mesh). Naphthalimide linked alkylamines (**2a**–**d**) [37,52], alcohol **4a** and bromide **5a** [40] were synthesized using reported methods.

5.1.1. 2-(2-Isothiocyanatoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (*3a*)

To a mixture of **2a** (0.5 g, 2.08 mmol) and potassium carbonate (0.87 g, 6.25 mmol) in chloroform (20 mL) was added thiophosgene (0.36 g, 3.12 mmol) drop wise over a period of 20 min. The mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The precipitate was removed by filtration, and the organic layer was washed with water and dried over $MgSO_4$. Removal of solvent under reduced pressure gave crude product which was purified through silica gel column chromatography using a mixture of ethyl acetate:hexanes (3:7), to yield (0.15 g, 26%) was pure **3a** as a white solid. Mp 214–215 °C. ^{1}H NMR (DMSO-d_6): δ 4.03 (t, 2H, J = 6.0 Hz), 4.39 (t, 2H, J = 6.0 Hz), 7.91 (dd, 2H, J = 8.0 and 7.5 Hz), 8.50 (dd, 2H, J = 8.5 and 1.0 Hz), 8.55 (dd, 2H, 7.0 and 1.0 Hz); ^{13}C NMR (DMSO-d_6): δ 39.2, 43.5, 122.4, 127.8, 128.0, 131.46, 131.8, 135.1, 164.0. HRMS (EI) m/z calcd for $C_{15}H_{10}N_2O_2S$, 282.0463; found: 282.0457.

5.1.2. 2-(2-Isothiocyanatobutyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (*3b*)

The compound **3b** was synthesized following a similar procedure as described above for **3a**. In brief, thiophosgene (0.32 g, 2.79 mmol) was added to a mixture of alkyl amine **2b** (0.5 g, 1.86 mmol) and K_2CO_3 (0.5 g, 3.73 mmol) in $CHCl_3$ (20 mL) and the mixture was stirred at room temperature for 2 h. Similar workup and purification as described for compound **3a** gave **3b** (0.3 g, 53%) as a white solid. Mp 159–160 °C. ^{1}H NMR ($CDCl_3$): δ 1.82–1.95 (m, 4H), 3.65 (t, 2H, J = 6.5 Hz), 4.27 (t, 2H, J = 7.0 Hz), 7.79 (dd, 2H, J = 8.0 and 7.5 Hz), 8.25 (d, 2H, J = 8.0 Hz), 8.63 (d, 2H, 7.0 Hz); ^{13}C NMR ($CDCl_3$): δ 25.3, 27.5, 39.2, 44.8, 122.5, 127.0, 128.2, 131.4, 131.6, 134.1, 164.3. HRMS (EI) m/z calcd for $C_{17}H_{14}O_2N_2S$, 310.0771; found: 310.0755.

5.1.3. 2-(2-Isothiocyanatohexyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (*3c*)

The compound **3c** was synthesized following a similar procedure as described above for **3a**. In brief, thiophosgene (0.437 g, 3.8 mmol) was added to a mixture of alkyl amine **2c** (0.75 g, 2.53 mmol) and K_2CO_3 (0.7 g, 5.06 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 2 h, filtered, and the solvent was evaporated to yield the crude mixture, which was purified by silica gel column chromatography, ethyl acetate:hexanes (3:7) to afford (0.66 g, 77%) of **3c** as a white solid. Mp 125–126 °C. ^{1}H NMR ($CDCl_3$): δ 1.46–1.55 (m, 4H), 1.72–1.81 (m, 4H), 3.54 (t, 2H, J = 5.5 Hz), 4.21 (t, 2H, J = 6.5 Hz), 7.78 (dd, 2H, J = 6.5 and 6.0 Hz), 8.23 (d, 2H, J = 6.5 Hz), 8.63 (d, 2H, 6.5 Hz); ^{13}C NMR (DMSO-d_6): δ 26.1, 26.2, 27.8, 29.6, 39.5–40.6 (1× $-CH_2-$, merged in DMSO peaks), 45.1, 122.5, 127.7, 127.8, 131.2, 131.8, 134.7, 163.9. HRMS (EI) m/z calcd for $C_{19}H_{13}O_2N_2S.Na$, 361.0981; found: 361.0965.

5.1.4. 3-Nitro-[2-(2-isothiocyanatoethyl)]-1H-benzo[de]isoquinoline-1,3(2H)-dione (*3d*)

The compound **3d** was synthesized following a similar procedure as described above for **3a**. In brief, thiophosgene (0.18 g, 1.57 mmol) was added to a mixture of **2d** (0.3 g, 1.05 mmol) and K_2CO_3 (0.29 g, 2.1 mmol) in 15 mL $CHCl_3$. The mixture was stirred at

room temperature for 2 h, filtered, and the solvent was evaporated to yield the crude mixture, which was purified by silica gel column chromatography, ethyl acetate:hexanes (3:7) to afford (0.16 g, 46.5%) of **3d** as a yellowish solid. Mp 212–213 °C. ^{1}H NMR (DMSO-d_6): δ 4.04 (t, 2H, J = 6.0 Hz), 4.40 (t, 2H, J = 6.0 Hz), 8.10 (dd, 1H, J = 8.0 and 7.5 Hz), 8.73 (dd, 1H, J = 7.5 and 1.0 Hz), 8.81 (dd, 1H, J = 8.5 and 1.0 Hz), 8.99 (d, 1H, J = 2.0 Hz), 9.52 (d, 1H, J = 2.0 Hz); ^{13}C NMR (DMSO-d_6): δ 39.5–40.6 (1× $-CH_2-$, merged in DMSO peaks), 43.4, 122.9, 123.5, 124.3, 129.8, 130.1, 130.4, 131.4, 134.6, 137.0, 146.3, 162.8, 163.3. HRMS (EI) m/z calcd for $C_{15}H_9O_4N_3S$, 327.0308; found: 327.0315.

5.1.5. 3-Nitro-[2-(2-hydroxyethyl)]-1H-benzo[de]isoquinoline-1,3(2H)-dione (**4b**)

A mixture 3-nitro-1,8-naphthalic anhydride (4.0 g, 16.46 mmol) and ethanolamine (3.01 g, 49.4 mmol) in EtOH (15 mL) was refluxed for 1 h. The reaction mixture was cooled to 4 °C, the solid formed was filtered, washed with EtOH (10 mL) and then with acetone and dried under reduced pressure to give 4 g (85%) of compound **4b** as a brownish solid. Mp 237–238 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.65 (dt, 2H, J = 6.5 and 6.0 Hz), 4.17 (t, 2H, J = 6.5 Hz), 4.38 (1, 1H, J = 6.0 Hz), 8.05 (dd, 1H, J = 8.0 and 7.5 Hz), 8.66 (dd, 1H, J = 7.5 and 1.0 Hz), 8.75 (d, 1H, J = 7.5 Hz), 8.93 (d, 1H, J = 2.0 Hz), 9.46 (d, 1H, J = 2.0 Hz). HRMS (EI) m/z calcd for $C_{14}H_{10}N_2O_5$, 286.0587; found: 286.0591.

5.1.6. 3-Nitro-[2-(2-bromoethyl)]-1H-benzo[de]isoquinoline-1,3(2H)-dione (**5b**)

Phosphorous tribromide (6.63 g, 24.4 mmol) was added drop wise to a solution of **4a** (3.5 g, 12.2 mmol) in ethyl acetate (50 mL) at 0 °C. After the addition was completed, the reaction mixture was heated at 80 °C for 2 h. The mixture was then cooled to RT and poured to ice cold water. The solid formed was filtered and purified by silica gel column chromatography using a mixture of methylene chloride:hexanes (1:1) to give 3.7 g (87%) of **5b** as a pale yellow solid. Mp 226–227 °C, ^{1}H NMR (DMSO-d_6): δ 3.69 (t, 2H, J = 7.5 Hz), 4.44 (t, 2H, J = 7.0 Hz), 8.05 (dd, 1H, J = 8.0 and 7.5 Hz), 8.69 (dd, 1H, J = 7.5 and 1.0 Hz), 8.78 (d, 1H, J = 8.5 Hz), 8.95 (d, 1H, J = 2.0 Hz), 9.47 (d, 1H, J = 2.0 Hz). HRMS (EI) m/z calcd for $C_{14}H_9O_4N_2Br$, 349.9725; found: 349.9723.

5.1.7. 2-[2-(Isothiourea)ethyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione (**6a**)

A mixture of **5a** (0.5 g, 1.7 mmol) and thiourea (0.11 g, 1.7 mmol) in acetonitrile (20 mL) was refluxed for 4 h. The reaction mixture was cooled, filtered and the precipitate was washed with ethyl acetate. The crude solid thus obtained was recrystallized from ethanol/diethyl ether to yield **6a** (0.45 g, 92%) as a white solid. Mp >285 °C. ^{1}H NMR (D_2O) δ 3.43 (t, 2H, J = 7.0 Hz), 4.37 (t, 2H, J = 7.0 Hz), 7.74 (dd, 2H, J = 8.0 and 7.5 Hz), 8.30 (dd, 2H, J = 8.0 and 7.5 Hz), 8.39 (dd, 2H, J = 7.0 and 1.0 Hz); ^{13}C NMR (DMSO-d_6): δ 29.1, 38.4, 122.3, 127.8, 127.9, 131.5, 131.8, 135.2, 164.1, 170.1. HRMS (EI) m/z calcd for $C_{15}H_{13}N_3O_2S$, 299.0728; found: 299.0734.

5.1.8. 3-Nitro-[2-{2-(isothiourea)ethyl}]-1H-benzo[de] isoquinoline-1,3(2H)-dione (**6b**)

A mixture of **5b** (0.54 g, 1.4 mmol) and thiourea (0.12 g, 1.5 mmol) in acetonitrile (20 mL) was refluxed for 4 h. The hot reaction mixture was filtered to remove insoluble reactant and the filtrate was evaporated to yield crude solid, which was titrated with hot ethyl acetate and filtered in hot condition to give pure **6b** (0.33 g, 62%) as a yellow solid. Mp 217–218 °C. ^{1}H NMR (DMSO-d_6): δ 3.73 (t, 2H, J = 7.0 Hz), 4.47 (t, 2H, J = 7.0 Hz), 8.08 (dd, 1H, J = 8.0 and 7.5 Hz), 8.71 (dd, 1H, J = 7.0 and 1.0 Hz), 8.81 (dd, 1H, J = 8.5 and 1.0 Hz), 8.98 (d, 1H, J = 2.0 Hz), 9.51 (d, 1H, J = 2.0 Hz). HRMS (EI) m/z calcd for $C_{15}H_{12}N_4O_4S$, 344.0579; found: 344.0583.

5.2. Cell lines and culture conditions

Human melanoma cell lines (UACC 903, A375-S2, SK-MEL-37 and 1205 Lu) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), in a humidified incubator at 37 °C with 5% CO_2.

5.2.1. MTS assay of cell proliferation

Human melanoma cell lines such as UACC 903, A375-S2, SK-MEL-37 and 1205 Lu (5× 10^3) were plated and grown for 24 h in 100 μL of growth medium in 96-well microtiter plates (Costar, Cambridge, MA) and then treated for 24 and 72 h with various concentrations of each compound, prepared from a 10 mM stock solution in DMSO. Control cells were treated with equivalent concentrations of DMSO alone. In all the cases, final concentrations of DMSO were 0.5%, well below the concentrations that interfere with proliferation in the above cell lines. After a 24 or 72 h incubation period, the number of viable cells was determined by measuring the bioreduction by intracellular dehydrogenases of the tetrazolium [41] compound MTS (Promega, Madison, WI) in the presence of the electron coupling reagent PMS. To perform the assay, 20 μL of combined MTS/PMS solution containing 2 mg/mL MTS and 0.92 mg/mL of PMS in PBS, pH 7.2 was added to each well, and the mixture was incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. Absorbance at 492 nm was measured using an ELISA microplate reader (Flexstation 3 Molecular Devices, Softmax Pro 5). Background absorbance of the medium was measured in a triplicate set of control wells that contained medium and the MTS/PMS solution without added cells and was subtracted from the absorbance measured in each of the sample wells to provide a corrected absorbance for each of the wells. Triplicate wells with predetermined cell numbers were subjected to the above assay in parallel with the test samples; this also provided internal confirmation that the assay was linear over the range of absorbance and hence, cell numbers measured. IC_{50} values were calculated by GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com) using data from three independent experiments.

5.2.2. Apoptosis determination by flow cytometry

Human melanoma cells UACC 903 were incubated at 37 °C for 24 h with different concentrations (1, 10 and 25 μM) of various naphthalimide derivatives or DMSO control. At the end of incubation, the cells were harvested by low speed centrifugation. In brief, the cells were washed twice with cold PBS and then resuspended in binding buffer at a concentration of 1 × 10^6 cells/mL. Cells were double stained by adding 7-AAD (5 μL) and PE Annexin V (5 μL) and incubated for 20 min at room temperature in the dark for detecting apoptosis following the instructions provided by the company (BD Biosciences). UACC 903 unstained cells were used as negative control; untreated and stained with 7-AAD and PE Annexin V cells were used as positive control. All these studies were conducted in three separate experiments. Discrimination (Fig. 3) of the four populations was validated by cell sorting; 7-AAD and Annexin V negative as live cells, 7-AAD positive as dead cells. Cells which were Annexin V positive and 7-AAD negative were identified as early apoptotic. Cells which were Annexin V positive and 7-AAD positive were identified as late apoptotic or necrotic.

5.3. Tumorigenicity assessment

Animal experimentation was performed according to protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University College of Medicine. Tumor kinetics were measured by subcutaneous injection of 5 × 10^6 UACC 903

melanoma cells in 0.2 mL of DMEM supplemented with 10% FBS above both left and right rib cages of 4–6 week old female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN). Six days later mice were randomly divided into control (DMSO) and experimental (**3a**, **3b**, **3c**, **3d**, **6a**, and **6b**) groups (5 mice/group; 2 tumors/mouse). Six days after subcutaneous injection of UACC 903 melanoma cells, mice were treated i.p. with ITC derivatives (0.6 μmole) or TU derivatives (0.5 μmole) three times per week (Monday, Wednesday and Friday). Control mice received an equal volume of the vehicle DMSO. The dimensions of the developing tumors (using calipers) and body weight were measured three times a week (Monday, Wednesday and Friday) and the size estimated in cubic millimeters.

5.4. Statistical analysis

Statistical analysis was undertaken using the one-way ANOVA followed by an appropriate *post hoc* test. Results were considered significant at a *P*-value of <0.05.

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