Radiosensitization of cancer cells by hydroxychalcones

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ABSTRACT

Radiation sensitization is significantly increased by proteotoxic stress, such as a heat shock. We undertook an investigation, seeking to identify natural products that induced proteotoxic stress and then determined if a compound exhibited radiosensitizing properties. The hydroxychalcones, 2′,5′-dihydroxychalcone (D-601) and 2,2′-dihydroxychalcone (D-501), were found to activate heat shock factor 1 (Hsf1) and exhibited radiation sensitization properties in colon and pancreatic cancer cells. The radiosensitization ability of D-601 was blocked by pretreatment with α-napthoflavone (ANF), a specific inhibitor of cytochrome P450 1A2 (CYP1A2), suggesting that the metabolite of D-601 is essential for radiosensitization. The study demonstrated the ability of hydroxychalcones to radiosensitize cancer cells and provides new leads for developing novel radiation sensitizers.

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Dietary polyphenols are present in fruits, vegetables, herbal medicines, wines, and spices. These compounds possess antioxidant, anti-inflammatory and anticancer activities. Chalcones are compounds with two aromatic rings and an unsaturated side chain, that is, phenyl ketone analogs of cinnamic acid. Hydroxychalcones are abundant in plant material and have been shown to have antitumor activities, which has been attributed to prooxidant phenoxyl radical formation rather than antioxidant properties.[1] It is the proteotoxic and genotoxic oxidative stress induced by hydroxychalcones that provide a rationale for their use as cancer chemotherapy. Radiation therapy represents an important tool for the treatment of colorectal cancer.[2,3] Its efficacy is a consequence of genotoxic oxidative stress. Current technology allows precise conformal 3-dimensional irradiation of a tumor, resulting in significant normal tissue dose sparing. Yet, even with this outstanding ability to physically target tumor tissue while sparing surrounding normal tissues, there are many instances in which tumors do not respond to irradiation. Furthermore, 8–10% of tumors that exhibit an initial response, recur and the median overall survival for these patients is approximately 2 years from the time of recurrence.[4,5] The failure of radiation therapy to yield definitive local-regional control underscores the urgent need for development of radiation sensitizers.

Heat shock (temperatures >42 °C) is one of the most potent radiation sensitizers identified to date.[6,7] Recent phase III clinical trials have demonstrated that hyperthermia can increase complete response rates and overall survival of patients with cervical tumors, as well as squamous and soft tissue sarcomas.[8–11] Hyperthermia, however, is currently limited clinically because of technical problems of heat delivery.[12] In order to overcome this problem, we designed and synthesized several novel indole analogs that enhance the degree of radiosensitization produced by a moderate heat shock (41 °C).[13,14] The molecular mechanisms involved in thermal radiosensitization involve a proteotoxic stress that initiates mitotic catastrophe and a late apoptosis.[13] We have also designed and synthesized several analogs that function as heat mimetics that produce radiosensitization at physiological temperatures.[15] In our continuing effort to develop heat mimetics that function as radiosensitizers at physiological temperature, we searched the scientific literature for proteotoxic compounds. In the present study two hydroxychalcones were selected for study. These compounds exhibit prooxidant properties, which are known to induce proteotoxic stress and initiate mitochondrial membrane depolarization.[1] The selection process also included commercial availability.

Hydroxychalcones (Fig. 1), 2′,5′-dihydroxychalcone (D-601) and 2,2′-dihydroxychalcone (D-501), were purchased from Indofine Chemical Company, Inc., NJ. Colony formation assays[15] of a human colon adenocarcinoma cell line (HT-29) and a human pancreatic carcinoma cell line (Panc-1) were used to estimate drug toxicity and radiation sensitivity. Cancer cells were treated with DMSO/drug for 2 h to determine non-toxic drug concentrations, defined as a plating efficiency[16] greater than 80%. After 10–14 days, cells were fixed for 15 min with 3:1 methanol/acetic acid and stained for 15 min with 0.5% crystal violet (Sigma) in methanol. Colonies were counted to estimate the percentage of survival. D-601 did not exhibit any toxicity (plating efficiency >80%) at 25 μM. D-501 was used at 10 μM (Plating efficiency >80%).

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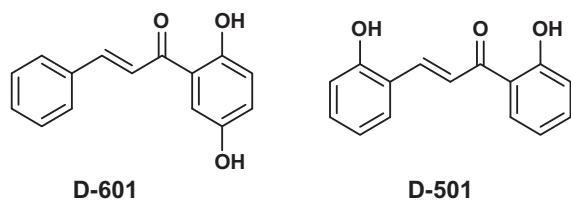
Figure 1. Structures of hydroxychalcones (D-601 and D-501) used in the present study. Compound numbers are their catalog number from Indofine Chemical Company.

D-501 (10 µM) was initially tested for its ability to sensitize HT-29 cells to mild heat shock (41 °C) (Fig. 2). D-501 exhibited robust thermal sensitization of HT-29 cells.

Destabilization of protein conformation by heat represents a common underlying biophysical mechanism for heat induced cell killing and thermal radiosensitization.[16–19] Enhanced Hsf1 DNA-binding activity is also observed with increased destabilization and unfolding of proteins.[20] Senisterra et al.[21] found that the minimum temperature that caused cellular proteins with intrinsically low conformational stability to unfold and denature is quantitatively the same minimum temperature required for Hsf1 activation. Hsf1 resides as a repressed monomer due to its association with Hsp90. In the presence of unfolded protein, Hsp90 disassociates from Hsf1, initiating Hsf1 trimerization and acquisition of DNA-binding activity.[20] We therefore used Hsf1 activation as a surrogate for measurement of chalcone-mediated global protein denaturation. HT-29 cells were treated with various concentrations of drug for 30 min and nuclear protein extracted. Gel shift assays were conducted using rat Hsp70 heat shock element (HSE) oligomer.[22] The data presented in Figure 3 illustrate significant enhancement of Hsf1 DNA binding activity upon addition of D-501 and D-601 (8 µM) to cells incubated for 2 h at 37 °C. HSF1 binding was not observed in control treatment. HSF1 supershift was conducted on Lane 10 using Hsf1 antibody confirming the presence of Hsf1 binding at the HSE. These results demonstrate that D-501 and D-601 destabilized protein conformation at physiological temperatures.

We determined if hydroxychalcones D-601 and D-501 (Data not shown) enhanced the radiation sensitivity of HT-29 and Panc-1 cells. Cells were exposed to drug/DMSO for 30 min/37 °C, during irradiation at room temperature and additional 90 min at 37 °C. Irradiation was accomplished using a Cs[137] source at 2 Gy per min. Cell survival was measured by clonogenic assay as described above. D-601 exhibited a concentration dependent radiosensitization of HT-29 cells (Fig. 4A). Full radiation dose response curves for HT-29 and Panc-1 cells are shown in Figure 4B and C. D-601 synergistically radiosensitized both cancer cell lines.
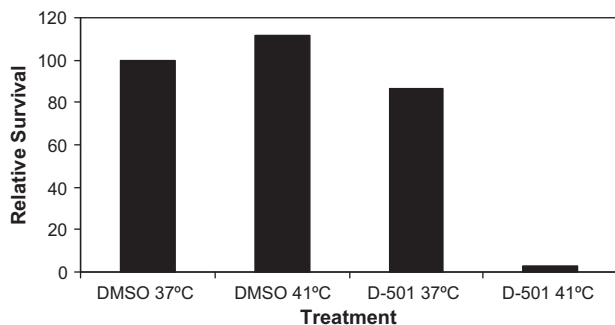
Figure 2. HT-29 cells were treated with D-501/DMSO and heat shocked at 41 °C for 2 h. Medium was changed and cells incubated for 10–14 days and then stained. Colonies were counted. Four flasks were used for each condition.

We also tested the potency of D-501. HT-29 cells were exposed to a dose of 10 µM D-501 and administered 4 Gy. Survival relative to the DMSO control was 0.43. Inspection of Figure 4A indicates that a dose of 20 µM D-601 was required to produce the same degree of radiation sensitivity. Thus, D-501 is twice as potent as D-601.

At the clinically relevant dose of 2 Gy, D-501 increased radiation sensitivity twofold. In HT-29 a dose of 2 Gy did not produce a statistically significant decrease in survival compared to non-irradiated cells (survival was 1.0 ± 2 Gy; $p > 0.05$; Student's t test). In the presence of 10 µM D-501, 2 Gy reduced survival to 0.53 ($p < 0.05$; Student's t test).

It is not known whether these chalcones exert their effects directly or through metabolites that may have formed due to P450 actions. Hydroxychalcones have been reported to possess cytotoxic properties.[1] Since P450 enzymes add hydroxy group to the hydroxychalcones,[23,24] it is hypothesized that metabolites of these chalcones could be the active substance. To test this hypothesis, we have used α-naphthoflavone (ANF) which is a specific inhibitor of CYP1A2 in combination with D-601. The results are presented in Figure 4D. Pretreatment of HT-29 cells with ANF partially reversed the effects of D-601 dependent radiosensitization. Pretreatment with a P450 3A inhibitor, miconazole did not affect the radiosensitization induced by D-601 (data not shown). These results support the hypothesis that a metabolite of D-601 formed due to the actions of cytochrome P450 1A2 is responsible for partial radiosensitization of HT-29 cells. We predict that D-501 also produces active metabolites due to its structural similarity to D-601. For example, hydoxylated metabolite of the catechol butein [2′,4′3,4-tetrahydroxychalcone] is more potent antioxidant, electron donor, and hydrogen donor than isoliquiritigenin [2,4,4′-trihydroxychalcone] due to the presence of additional hydroxy group.[25] We hypothesize that the increased potency of D-501 is due to multiple hydroxylations of D-501 (due to activation of both rings by hydroxy groups present in the molecule) by P450 enzyme(s). This could lead to formation of diquinone product as shown with diphenolic compounds[26] and ultimately generate oxidative stress.

Cell cycle analysis was performed in the presence of D-601 and radiation. Cells were treated with DMSO/drug for 2 h and were harvested. The cells were suspended in 500 µl of PBS containing 0.1% glucose and kept at 4 °C. Cold 70% EtOH (5 ml) was immediately added and mixed. Prior to FACS analysis, the cells were washed with PBS, added 300 µl of propidium iodide solution and 20 µl of 10 mg/ml RNase. The contents were incubated at 37 °C for 30–45 min. The cells were transferred to FACS tubes and analyzed. The analysis demonstrated that exposure to D-601 plus radiation induced polyploidy formation that was followed by an apoptotic response, as measured by sub-G_0 cells 48 h after irradiation (data not shown).

In the present study, we have demonstrated the ability of hydroxychalcones to induce proteotoxicity at physiological temperatures. Based on our previous studies,[13] we hypothesize that radiosensitization is a consequence of denaturation of critical proteins by D-601 that leads to mitotic catastrophe and a late apoptotic response. Chalcones are known to exhibit a wide variety of activities including glutathione depletion which leads to Phase II enzyme induction through activation of transcription factor, Nrf2. This is the basis of cancer prevention studies. In this case, chalcones act as Michael Reaction Acceptors due to the presence of α,β-unsaturated ketone.[27] We have shown that glutathione depletors activate heat shock factor 1 (Hsf1) and induce HSP70 protein expression.[28] Thermal sensitization was not observed when indole based chalcones were tested (data not shown). Thus, we predict the thermal sensitization property of hydroxychalcones is not completely based on their glutathione depletion properties. Further study is needed to identify the exact mechanism by which hydroxychalcones function as radiation sensitizers. In addition,
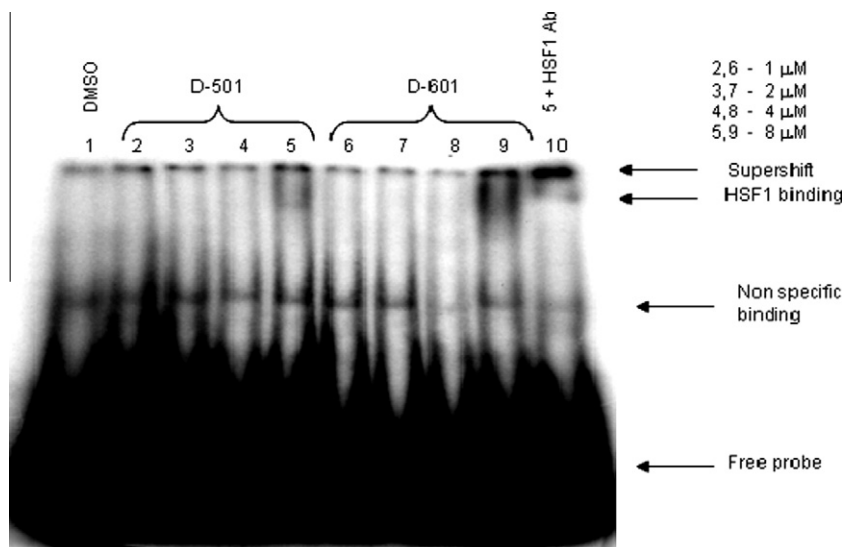
Figure 3. Gel mobility shift assay. HT-29 cells were treated with D-501 and D-601 at various concentrations and nuclear protein was probed for binding to ^{32}P labeled heat shock element (HSE).
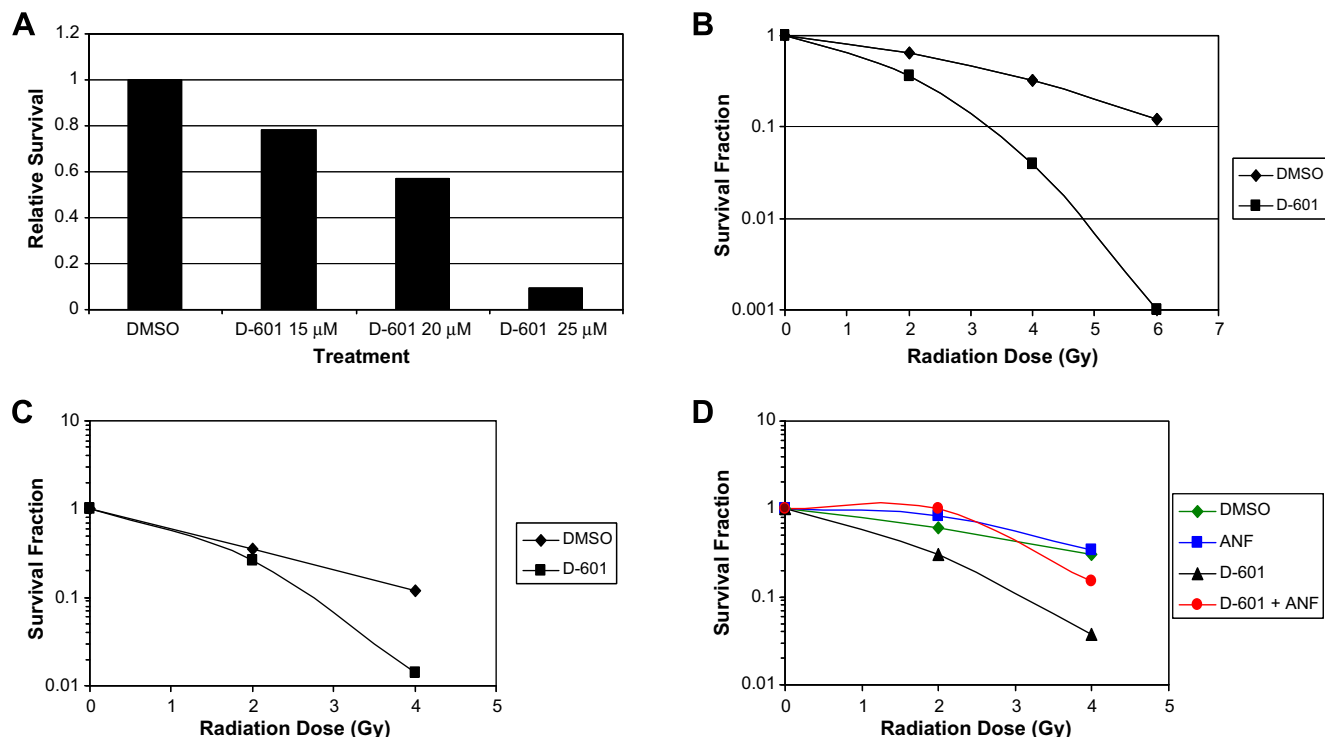
Figure 4. Radiosensitization of HT-29 and Panc-1 cells by D-601. (A) Concentration effect of D-601 on HT-29 cell survival following 4 Gy. Survival following 4 Gy (DMSO control) was 0.25 and is expressed as a relative survival of 1.0; (B) survival of HT-29 cells at 25 μM of D-601 and various radiation doses; (C) effect of D-601 and radiation on the survival of Panc1 cells; (D) effect of α-napthoflavone (ANF) on radiosensitization by D-601.

we have shown that P450 enzyme inhibitor partially blocks radio-sensitization generated by D-601 treatment. Guo et al.[24] have shown that hydroxychalcone, isoliquiritigenin, generated several metabolites which include hydroxylated product (2′,4,4′,5′-tetra-hydroxychalcone), cyclized products (4,4′-dihydroxyflavanone), and cis- and trans-6,4′-dihydroxyaurone. In the present study we have not studied which metabolites formed from D-601. We predict a minimum of hydroxylated product and cyclized flavanone formation with this drug. Further study needed to identify active metabolite. In conclusion, these compounds provide new leads for the development of novel thermal radiosensitizers that function at physiological temperature.

Acknowledgements

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