Novel Chemical Enhancers of Heat Shock Increase Thermal Radiosensitization through a Mitotic Catastrophe Pathway

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Abstract

Radiation therapy combined with adjuvant hyperthermia has the potential to provide outstanding local-regional control for refractory disease. However, achieving therapeutic thermal dose can be problematic. In the current investigation, we used a chemistry-driven approach with the goal of designing and synthesizing novel small molecules that could function as thermal radiosensitizers. (*Z*)-(±)-2-(1-Benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol was identified as a compound that could lower the threshold for Hsf1 activation and thermal sensitivity. Enhanced thermal sensitivity was associated with significant thermal radiosensitization. We established the structural requirements for activity: the presence of an *N*-benzenesulfonylindole or *N*-benzylindole moiety linked at the indolic 3-position to a 2-(1-azabicyclo[2.2.2]octan-3-ol) or 2-(1-azabicyclo[2.2.2]octan-3-one) moiety. These small molecules functioned by exploiting the underlying biophysical events responsible for thermal sensitization. Thermal radiosensitization was characterized biochemically and found to include loss of mitochondrial membrane potential, followed by mitotic catastrophe. These studies identified a novel series of small molecules that represent a promising tool for the treatment of recurrent tumors by ionizing radiation. [Cancer Res 2007;67(2):695–701]

Introduction

The use of ionizing radiation is essential for the management of many human cancers. Technological advancements allow achievement of outstanding physical targeting of ionizing radiations to tumor tissue, sparing normal tissue and providing the potential for definitive local-regional control. Local-regional control is the primary requirement for preventing progression to devastating invasive disease. Unfortunately, local-regional control is not always obtained in many cases of aggressive disease. Thus, there is an urgent need for development of radiation sensitizers.

Therapeutic hyperthermia is one of the most potent radiation sensitizers identified to date (1). Radiosensitization produced by therapeutic hyperthermia is a consequence of heat shock simultaneously affecting protein dynamics in multiple pathways, including the response to radiation-induced DNA double-strand breaks (2). Clinical trials have shown that when therapeutic hyperthermia is used as an adjuvant with ionizing radiation, significant improvement in local-regional control can be achieved (3, 4). Phase III clinical studies established that radiation therapy combined with adjuvant therapeutic hyperthermia can produce significant local control in disease refractory to ionizing radiation (4, 5). However, for many cancers, suboptimal thermal doses limit radiosensitization (6). Thus, small molecules designed to enhance hyperthermia have the potential to improve radiotherapy outcomes.

Consideration of the mechanisms by which heat shock induces radiosensitization offers an opportunity for innovative chemistry-driven drug discovery of small molecules that convert subtherapeutic hyperthermic exposures into efficacious thermal doses that yield robust radiosensitization. The foremost molecular event governing thermal radiosensitization is the unfolding and aggregation of a subset of cellular proteins (2). Heat shock–mediated alterations of the biophysical properties of irradiated chromatin, temperature-dependent inhibition of DNA double-strand break ligation (1), and the usurping of signal transduction pathways are mainly a consequence of protein unfolding and aggregation (7). Thus, thermal destabilization of protein conformation represents an underlying biophysical process that can be exploited.

Materials and Methods

Synthesis of indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols. Indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols and related non-indolic compounds were prepared by aldol condensation of the appropriate *N*-substituted indole-3-carboxaldehydes/arylcarboxaldehyde with 1-azabicyclo[2.2.2]octan-3-one to afford the corresponding *N*-substituted indol-3-ylmethylene/arylmethylene-1-azabicyclo[2.2.2]octan-3-one. The 3-keto analogue could be reduced to the corresponding indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene/arylmethylene)-1-aza-bicyclo[2.2.2]octan-3-ol with sodium borohydride in methanol.

Assays. Hyperthermia was done in temperature-controlled water baths. A Pantak orthovoltage X-ray machine was used to irradiate cells at 2.05 Gy/min (300 kVp). Colony formation was used to quantitate cell survival.

Fluorescence recovery after photobleaching. The nuclear mobility of Mre11-GFP in a stably transfected HCT116-Mre11-GFP fusion protein cell line was measured by a fluorescence recovery after photobleaching methodology (FRAP), using a Zeiss LSM510 inverted confocal fluorescence microscope. Please see Supplementary Methods for a detailed description.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-3212

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Gel mobility shift assays, determination of mitochondrial membrane potential, fluorescence-activated cell sorting analysis, and 53BP1 immunofluorescence. Gel mobility shift assays were done as described in (8). Mitochondrial membrane potentials were determined by fluorescence-activated cell sorting analysis using the fluorescent dye JC-1 (Molecular Probes/Invitrogen, Carlsbad, CA). 53BP1 immunofluorescence was determined in MCF7 cells using a monoclonal antibody against 53BP1 that was a gift from Dr. J. Chen (Yale University School of Medicine, New Haven, CT).

Results and Discussion

(*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols (IMZO-3-ol) function as enhancers of thermal dose. Indomethacin is a nonsteroidal anti-inflammatory drug shown to lower the temperature required for initiation of the heat shock response (9, 10) and the threshold temperature for hyperthermic radiosensitization (10). Thus, the *N*-benzoylindole moiety of the indomethacin molecule represented a structural platform upon which a chemistry-driven synthesis approach could be initiated. A series of novel indole-*N*-substituted IMZO-3-ols, their corresponding 3-keto analogues, and other structurally related non-indolic compounds were synthesized (Fig. 1) and were structurally characterized by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, gas chromatography-mass spectroscopy analysis, and elemental combustion analysis. X-ray analysis was used to identify the molecular geometry, conformation, stereochemistry, and atom connectivity of two representative analogues (see Fig. 1, *V* and *VI*; ref. 11). The indole rings in these two compounds are planar, and the benzene ring of the benzenesulfonyl group makes a dihedral angle of 90.2 degrees (compound V) or 94 degrees (compound VI) with the mean plane of the indole ring. In both analogues, the double bond connecting the azabicyclic and indole moieties has the *Z* geometry.

HT29 colon carcinoma cells, which are intrinsically resistant to heat shock (12), were used as a model in a comparative structure-activity analysis. In the initial experiments, cells were exposed to 150 μmol/L of compounds I to V (Fig. 1) for 2 to 4 h at either 37°C or 41°C. At 37°C, compounds I to V were nontoxic: the plating efficiency (13) was not statistically significant from control ($P > 0.05$, Student's t test; data not shown). These initial experiments identified (*Z*)-(±)-2-(1-benzenesulfonyl-indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol (compound V) as a potent enhancer of thermal dose. Whereas a 2-h/41°C thermal treatment was nontoxic, as measured by colony formation (Supplementary Fig. S1*A*; $P > 0.05$ Student's t test), incubation in the presence of compound V at this temperature significantly increased thermal sensitivity ($P < 0.05$, Student's t test).

Hyperthermic radiosensitization is directly related to thermal sensitization. Thus, the thermal-sensitizing properties of compound V produced significant thermal radiosensitization, yielding a thermal enhancement ratio of ~3, calculated at a survival level of 50% (Fig. 2*A*; Supplementary Fig. S1*B*; $P < 0.05$, Student's t test). In contrast, radiation sensitivity was not affected by noncytotoxic treatments consisting of a 41°C exposure in the absence of enhancer, exposure to compound I or IV during thermal treatment, or exposure to enhancer at 37°C before x-irradiation (Supplementary Fig. S1*B*; $P > 0.05$, Student's t test). Similar results were obtained in MCF-7 and H460 cells (data not shown).

Hyperthermic doses capable of producing radiosensitization induce a fraction of nuclear Mre11, a component of the Mre11-Nbs1-Rad50 DNA double-strand break sensing complex, to translocate to the cytosol (14, 15). Using FRAP, we have further observed that the fraction of Mre11 that remains in the nucleus during therapeutic hyperthermia undergoes a progressive heat-dependent loss of mobility. This was shown using HCT116 cells

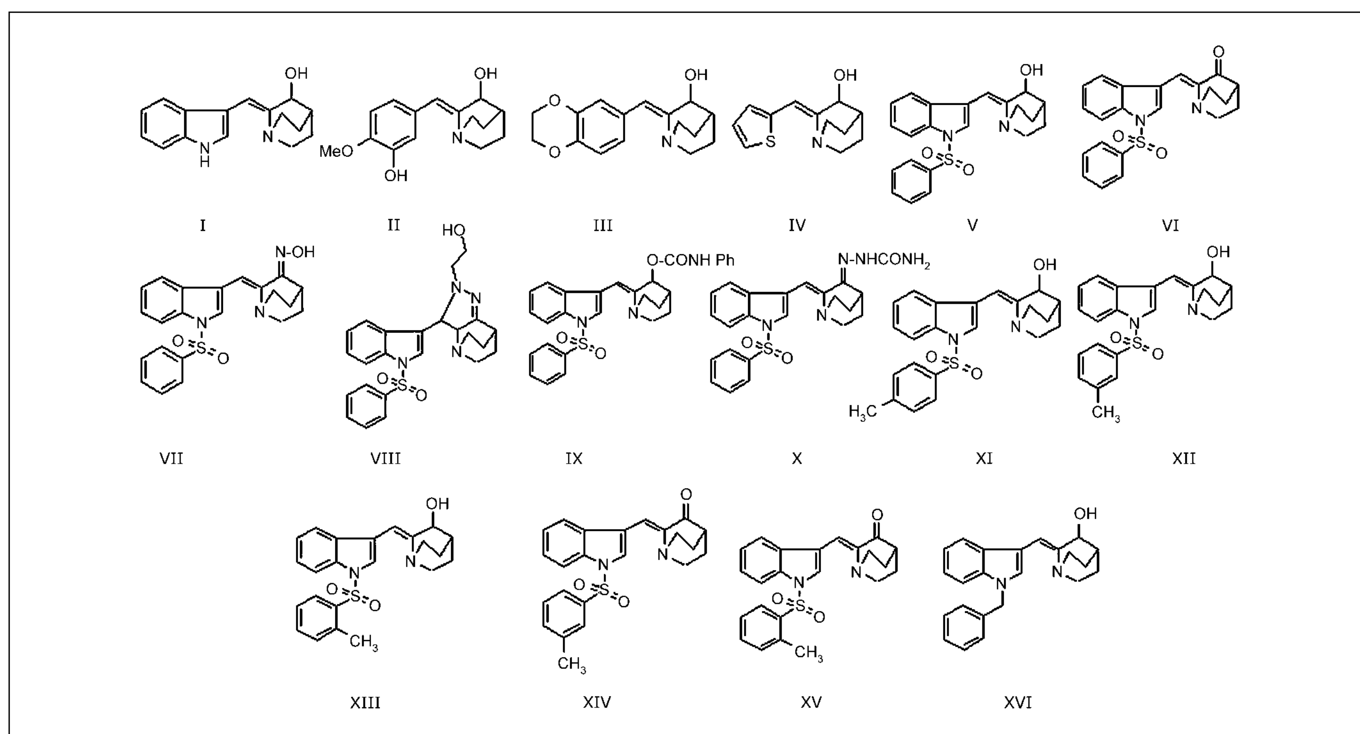

Figure 1. Structures of a series of novel indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols and their 3-keto analogues together with other structurally related non-indolic compounds.

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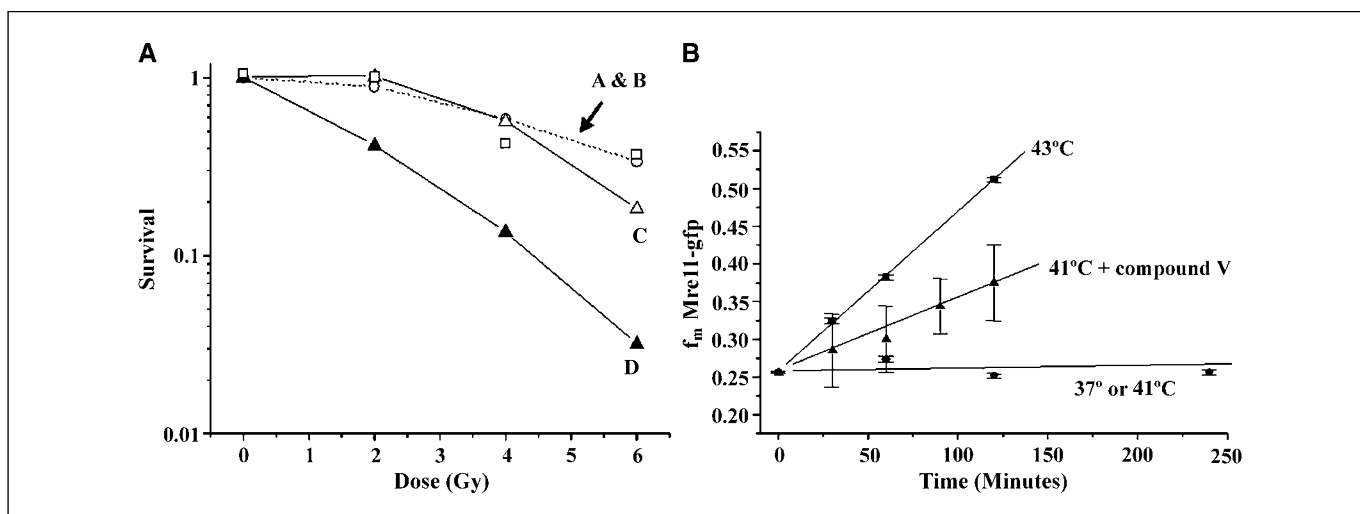

Figure 2. (*Z*)-(±)-2-(1-Benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol (compound V) functions as a small-molecule enhancer of thermal radiosensitization. *A,* HT-29 cells were incubated at either 37°C (*curves A* and *B*) or 41°C (*curves C* and *D*) for 4 h in the absence (*curves A* and *C*) or presence (*curves B* and *D*) of 150 μmol/L (*Z*)-(±)-2-(1-benzenesulfonyl-indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol (*curves B* and *D*) incubated for 10 min at room temperature and then x-irradiated with the indicated doses at room temperature. Cells were immediately washed, and survival was determined by measuring colony-forming ability 10 to 15 days later. Cells designated as control were exposed to DMSO as a vehicle control. A thermal enhancement ratio was calculated at a survival value of 50%. *B,* normalized FRAP curves of nuclear Mre11-GFP were obtained at 37°C, 41°C, and at 43°C. Fluorescence intensity measurements. *Points,* average of 5,000 measurements taken over ~6 ms; *bars,* SE. Measurements were taken 25 times for each condition.

stably expressing a Mre11-GFP fusion protein. This Mre11-GFP chimera functions similar to endogenous Mre11 in terms of subcellular localization and response to irradiation (Supplementary Fig. S2*A* and *B*). Thus, heat-mediated immobilization of nuclear Mre11 can be used as a molecular signature of thermal radiosensitization.

Compound V enhanced heat-mediated Mre11-GFP chimera immobilization in HCT-116 cells. Whereas, a 41°C thermal pretreatment yielded a Mre11-GFP FRAP mobility curve that was indistinguishable from the 37°C control curve (Supplementary Fig. S3), a significant decrease in mobility was observed following a 43°C thermal treatment (Fig. 2*B*; Supplementary Fig. S3). Addition of compound V (0.25 mmol/L) to HCT116 Mre11-GFP fusion cells incubated at 41°C significantly decreased Mre11-GFP mobility, to a similar degree as was observed in the samples treated at 43°C alone. The relationship between the immobile Mre11-GFP fraction and time at hyperthermic temperatures is illustrated in Fig. 2*B*. We conclude that the addition of an indole-*N*-substituted IMZO-3-ol to a 41°C thermal treatment converted a thermal dose that was subeffective in altering Mre11 mobility into one that was capable of inhibiting Mre11 mobility. Taken altogether, the data presented in Fig. 2 indicate that compound V can function as a small-molecule enhancer of thermal radiosensitization and is significantly more effective than indomethacin in that it produces enhancement at a lower temperature and at lower concentration (10).

Systematic structural modification of the 1-azabicyclo[2.2.2]octan-3-ol moiety was undertaken (Fig. 1; Supplementary Fig. S4*A*). Oxidation of the hydroxyl group to a 3-keto group improved enhancer activity (*VI* versus *V*: $P < 0.05$, Student's t test), whereas other structural modifications resulted in loss of enhancer activity (compounds VII–X). Introduction of a methyl substituent into the aromatic ring of the *N*-benzenesulfonyl moiety (compounds XI–XV) also caused a loss of activity. The ability of molecules containing an indole *N*-benzenesulfonyl moiety (compound V) to function as an enhancer of a 2-h/41°C thermal treatment was compared with molecules containing an *N*-benzylindole moiety (XVI). Substitution of the *N*-benzenesulfonyl group in compound V for an *N*-benzyl moiety (XVI) increased enhancer activity by 50% ($P < 0.05$, Student's t test; data not shown). The *N*-benzylindole thermal sensitizing analogue (XVI) was also an effective thermal radiosensitizer (Supplementary Fig. S5).

Thermal destabilization of protein is enhanced in the presence of an indole-*N*-substituted IMZO-3-ol. Thermal-mediated destabilization of protein conformation represents a common underlying biophysical mechanism for hyperthermic-induced cell killing and thermal radiosensitization (2, 7, 16, 17). Protein destabilization and unfolding also initiates the acquisition of Hsf1 DNA-binding activity (18). Senisterra et al. (19) found that the minimum temperature required for Hsf1 activation was quantitatively the same as the minimum temperature that caused cellular proteins with intrinsically low conformational stability to unfold and denature. 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 (18). We therefore used Hsf1 activation as a surrogate for measurement of (*Z*)-(±)-2-(1-benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol–mediated global protein denaturation. The data presented in Fig. 3*A* and *B* illustrate significant enhancement of Hsf1 DNA-binding activity upon addition of compound V to cells incubated at 41°C for 2 or 4 h. Note that addition of an Hsf1 antibody retarded DNA-binding activity, whereas addition of excess unlabeled HSE oligonucleotides competed away DNA-binding activity (Fig. 3*A*). Increased expression of Hsp70 mRNA was observed only upon addition of compound V or the structurally related compound XVI to a 4-h/41°C heat shock (Fig. 3*C*). Similarly, addition of compound V increased translation of Hsc/Hsp70 (Fig. 3*D*).

The relationship between conformational stability of a protein and the temperature of its environment are integrally related.

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Figure 3. Indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols function as enhancers of the heat shock response. *A* and *B,* gel mobility shift assay analysis of HT29 nuclear cell extracts (10 μg per lane) done with HSE ds oligonucleotides. HT29 cells were incubated at the specified temperatures for the indicated times in the absence or presence of 150 μmol/L of analogue V in Fig. 1, washed extensively, and incubated at 37°C in the absence of enhancer for 4 h before obtaining nuclear lysates. Hsf1 antibody was added to some of the lysates. A 50-fold excess of cold competitor was added to other extracts. *C,* Northern blot of mRNA isolated from HT29 cells incubated at 37°C or 41°C for 4 h in the absence or presence of 150 μmol/L analogue V or XVI (Fig. 1), washed extensively, and incubated at 37°C in the absence of enhancer for 4 h before isolating mRNA. *D,* immunoblot for Hsc/Hsp70. HT 29 cells were exposed to 150 μmol/L analogue V while incubated at 37°C or 41°C for 4 h. The cells were then washed, held at 37°C for 4 h before isolating cellular protein, and subjected to immunoblotting with antibody that recognizes Hsc and Hsp70.

At physiologic temperatures, a protein resides in a spectrum of natively folded states that exhibit varying degrees of stability and that are only slightly more stable than unfolded conformations (20). As temperature is increased, random thermal fluctuations away from the most compact conformations (21) increase the probability of transition to an unfolded state or states. This can be measured using differential scanning calorimetry (DSC), a procedure that detects heat absorbed as proteins unfold. In living cells, this technique provides a direct physical measurement of protein destabilization and unfolding, yielding scans of excess specific heat (C_p) versus temperature (2). Analysis of DSC profiles provides support the hypothesis that 2-(indol-3-ylmethylene)quinuclidines can function as small molecules that enhance thermally mediated protein denaturation (Supplementary Fig. S6).

The conformational stability of a protein can be decreased by the binding of small molecules (22). The demonstration of a stringent structural requirement for thermal enhancement of cell killing, the observation that indole-*N*-substituted (*Z*)-(±)-2-(indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ols function to lower the temperature required for Hsf1 activation, coupled with the DSC analysis suggest the hypothesis that these small molecules recognize and associate with amino acid motifs present in many cellular proteins, thereby decreasing the conformational stability of a subset of cellular proteins and initiating thermal sensitivity. To this end, we used compound XVI as bait in a peptide library screen. Screening of the peptides against the Prosite Motif database revealed a potential binding motif that is present in numerous proteins.[6]

Pathway identification. Heat shock–mediated denaturation of key proteins in critical pathways affects many cellular functions. We surveyed several pathways known to be affected by heat shock and irradiation (23–29). Loss of mitochondrial membrane potential ($\Delta\psi_m$), an important initiator of cell death signaling (30, 31), can be a consequence of heat shocks capable of denaturing protein and activating Hsf1 (23). HT29 cells were incubated for 4 h at either 37°C or 41°C in the presence or absence of compound V. Cells were then subjected to a flow cytometric bivariate analysis of JC-1 fluorescence, which is presented as the change in red fluorescent, indicative of polarized mitochondrial membranes, versus green fluorescent, representing depolarized mitochondria (Fig. 4). The red to green emission fluorescence intensities yielded values of 1.4 and 1.1 in cells held at 37°C in the absence and presence of enhancer, respectively. In cells incubated at 41°C, the values were 1.4 in the absence of enhancer and 0.2 in the presence of enhancer. The shift to green fluorescence, observed in the entire population of cells heated in the presence of compound V, indicates that a significant loss of mitochondrial membrane potential ($\Delta\psi_m$) occurred in these cells. Taken altogether, the data are consistent with the hypothesis that drug-mediated thermal sensitization and

[6] K.R. Sekhar, unpublished preliminary data.

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hyperthermic radiosensitization are directly related to the ability of compound V to initiate a loss of $\Delta\psi_m$ during heat shock.

The clonogenic survival assays shown in Fig. 2 yield little mechanistic information concerning cell death pathways (32). Apoptosis, necrosis, mitotic catastrophe, post-mitotic cell death, and senescence can all contribute to a loss of clonogenicity in heated and irradiated cells (32, 33). Mitotic catastrophe events, defined as cell death occurring as a result of a deregulated or failed mitosis (34), can follow loss of $\Delta\psi_m$ (29). Mitotic catastrophe can be distinguished by formation of multinucleated and/or giant cells (34, 35) that contain sub-G_1 DNA (36). Examples of the morphologic criteria used in this investigation are shown in Supplementary Figs. S7 and S8. We found that neither a 4-h/41°C thermal exposure by itself nor exposure to the small-molecule enhancer at 37°C for 4 h induced multinuclei or giant cell formation, whereas both were observed in cells incubated at 41°C in the presence of enhancers V, VI, and XVI. Representative results are illustrated below.

A 10-fold increase in multinucleated cells was observed 48 h following a 4-h/41°C exposure to compound VI (Table 1A; $P < 0.05$, Student's t test), an enhancer that produced a significant increase in thermal sensitivity (Supplementary Fig. S4), yet was not cytotoxic to cells held at 37°C (data not shown). The frequency of multinucleation was not significantly affected if cells were incubated at 41°C in the absence of enhancer or at 37°C in the presence of enhancer ($P > 0.05$). Similar results were observed in MCF7 cells (data not shown). Multinuclear events increased 40% compared with irradiation alone, when cells were exposed to a 2-h/41°C thermal treatment in the presence of compound XVI 10 min before administration of 6 Gy ($P < 0.05$ Student's t test). Neither the thermal treatment given alone nor exposure to compound XVI at 37°C significantly increased multinuclear events following irradiation ($P > 0.05$ Student's t test; Table 1A).

Multinucleated cells that undergo mitotic catastrophe contain significant levels of sub-G_1 DNA (36). We found that the presence of an indole-*N*-substituted IMZO-ol-3-one (e.g., compound VI) increased the frequency of sub-G_1 DNA in cells exposed to a 4-h/41°C heat shock and in cells exposed to this heat shock plus 6 Gy (2.5- to 3-fold compared with control; $P < 0.05$, Fisher's exact test; Table 1B). However, Annexin V-FITC/propidium iodide staining of HT29 cells incubated at 41°C for 4 h in the presence of enhancer was not significantly different from that observed in control cells held at 37°C in the

Table 1.

A. (*Z*)-(±)-2-(1-Benzenesulfonyl-indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-one (compound VI) and (*Z*)-(±)-2-(1-benzylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol (compound XVI) enhance mitotic catastrophe

Temperature	Dose	Structure	Multinuclear/total	% Multinuclei
37°C/4 h	0	—	7/801	0.9
	0	VI	3/363	0.8
41°C/4 h	0	—	2/388	0.5
	0	VI	20/194	10
37°C/2 h	6 Gy	—	34/234	15
	6 Gy	XVI	12/220	5
41°C/2 h	6 Gy	—	13/102	13
	6 Gy	XVI	21/100	21

B. (*Z*)-(±)-2-(1-Benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-one (compound VI) enhances formation of sub-G_1 DNA content

Temperature	Dose	Structure	% Sub-G_1	% G_1	% S	% G_2
37°C/4 h	0	—	1.2	68	17	13
	0	VI	0.8	69	17	12
41°C/4 h	0	—	1.3	69	16	13
	0	VI	4	63	20	11
37°C/4 h	6 Gy	—	3.5	60	17	18
	6 Gy	VI	2.8	58	18	20
41°C/4 h	6 Gy	—	5.1	54	22	18
	6 Gy	VI	9	52	17	19

NOTE: *A,* HT-29 cells were incubated at either 37°C or 41°C in the absence or presence of 150 μmol/L of the indicated enhancer, immediately cooled to room temperature, incubated for 10 min before administration of 0 or 6 Gy, washed extensively, and incubated at 37°C for 48 h. Multinuclear cells were counted by phase-contrast microscopy and confirmed using criteria described in Supplementary Fig. S8. *B,* HT-29 cells were incubated at either 37°C or 41°C for 4 h in the absence or presence of 150 μmol/L of compound VI, incubated for 10 min at room temperature, and then x-irradiated with 0 or 6 Gy. Cells were immediately washed and incubated at 37°C for 48 h, and cell cycle profiles analyzed by propidium iodide staining of DNA content using fluorescence-activated cell sorting analysis.

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absence of enhancer, measured 4 or 48 h after heat shock (data not shown). The observations that mitotic catastrophe occurred in cells lacking phosphatidylserine exposure is consistent with the work of Zhang et al. (32). Taken altogether, these results support the hypothesis that indole-*N*-substituted IMZO-ols and IMZO-3-ones function as small-molecule enhancers of thermal sensitivity and thermal radiosensitization by shifting cells into a mitotic catastrophe pathway.

The loss of 53BP1 function is associated with both mitotic catastrophe and sensitivity to ionizing radiation (37, 38). Therefore, irradiation-induced 53BP1 containing foci formation was used to interrogate 53BP1 function in cells incubated at 41°C for 4 h and then exposed to 2 Gy. Foci formation was moni tored 0.5 to 8 h after irradiation in MCF 7 cells, as their optical qualities are superior to HT29 cells. As stated above, compound V enhanced the thermal sensitivity of MCF7 cells (data not shown).

We observed a 10-fold increase in radiation-induced 53BP1 foci 30 min after cells received 2 Gy (Supplementary Fig. S9). This increase takes into account foci present in unirradiated cells. This level of radiation-induced foci was sustained for an additional 5.5 h, returning to control values 8 h after irradiation (data not shown). A 4-h/41°C heat shock did not affect radiation-induced 53BP1 foci formation (Supplementary Fig. S9). Cells exposed to compound V during a 4-h/41°C heat shock before administering 2 Gy exhibited a significant alteration in the appearance of 53BP1 foci (Fig. 5). Two morphologies were observed: a majority of the cells exhibited a dim fluorescent pattern with lack of discrete radiation induced foci. A few cells exhibited an intense 53BP1-mediated congealed fluorescent signal. These changes were not observed in cells exposed to compound V at 37°C, where normal

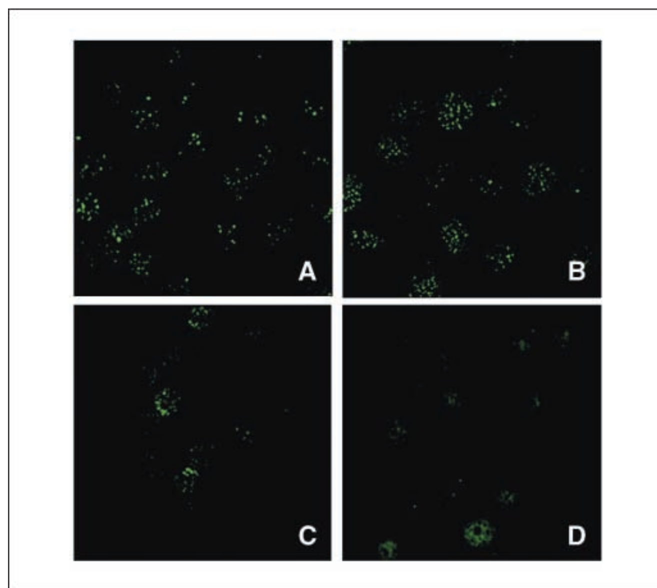

Figure 5. Exposure to 150 μmol/L (*Z*)-(±)-2-(1-benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol during a heat shock altered radiation-induced 53BP1 foci formation. Equal numbers of MCF7 cells were held at exposed to 150 μmol/L (*Z*)-(±)-2-(1-benzenesulfonyl-indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol for 4 h at either 37°C (*A* and *B*) or 41°C (*C* and *D*). Some of the cells were then irradiated at room temperature with 2 Gy (*B* and *D*). One hour after irradiation, the cells were fixed and stained with 53BP1 antibody.

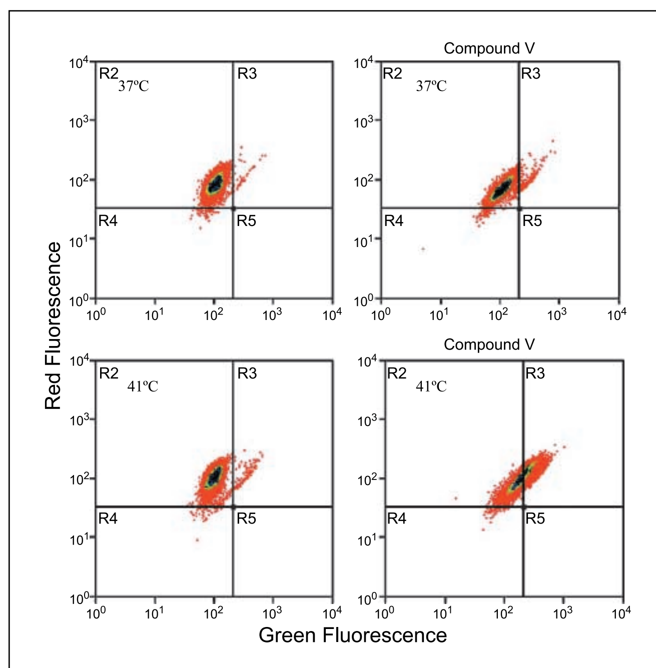

Figure 4. Loss of mitochondrial membrane potential ($\Delta\psi_m$) in HT29 cells exposed to 41°C in the presence of compound V. HT29 cells were incubated for 4 h at either 37°C or 41°C in the presence or absence of 150 μmol/L of enhancer. Cells were then subjected to a flow cytometric bivariate analysis of JC-1 fluorescence.

radiation-induced 53BP1 foci were observed. Thus, radiation induced 53BP1 foci formation represents an additional pathway affected by a (*Z*)-(±)-2-(1-benzenesulfonylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol, consistent with a progressive increase in mitotic catastrophe and increased radiation sensitivity observed in this cell population.

In summary, we have exploited a chemistry-driven drug discovery program for the purpose of developing small molecules that could enhance the underlying biophysical and biochemical pathways affected by heat shock that contribute to thermal sensitivity and thermal radiosensitivity. This approach identified (*Z*)-(±)-2-(1-benzenesulfonyl-indol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol (compound V) as a small molecule that could function as a thermal enhancer and established the structural requirement for the presence of an *N*-benzenesulfonyl-indole or *N*-benzyl-indole moiety linked at the indolic 3-position to a 2-(1-azabicyclo[2.2.2]octan-3-ol) or 2-(1-azabicyclo[2.2.2]octan-3-one) moiety. The synthesis of these small molecules represents a promising tool for the clinical application of therapeutic hyperthermia as a radiation sensitizer for the treatment of recurrent tumors. As these molecules enhance multiple thermal effects, it may be hypothesized that these agents have the potential to enhance additional cancer therapies augmented by hyperthermia, such as chemotherapy and immunotherapy (39).

Acknowledgments

Received 8/30/2006; revised 10/27/2006; accepted 11/20/2006.

Grant support: NIH/National Cancer Institute grant PO1 CA104457.

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Novel Chemical Enhancers of Heat Shock Increase Thermal Radiosensitization through a Mitotic Catastrophe Pathway

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Cancer Res 2007;67:695-701.

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