Antiangiogenic properties of substituted (Z)-(±)-2-(N-benzylindol-3-ylmethylene)quinuclidin-3-ol/one analogs and their derivatives

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ABSTRACT

In the past half century research efforts have defined a critical role for angiogenesis in tumor growth and metastasis. We previously reported that inhibition of a novel target, ENOX1, by a (Z)-2-benzylindol-3-ylmethylene) quinuclidin-3-ol, suppressed tumor angiogenesis. The present study was undertaken in order to establish structure–activity relationships for quinuclidine analogs. The angiogenesis inhibiting activity of a series of substituted (Z)-(±)-2-(N-benzylindol-3-ylmethylene)quinuclidin-3-ols (**1a–1k**), (Z)-2-benzylindol-3-ylmethylene)quinuclidin-3-ones (**2a–2h**), (Z)-(±)-2-(1H/N-methyl-indol-3-ylmethylene)quinuclidin-3-ols (**3a–3b**), and substituted (Z)-(±)-2-(N-benzenesulfonylindol-3-yl-methylene) quinuclidin-3-ols and their derivatives (**4a–4d**) that incorporate a variety of substituents in both the indole and N-benzyl moieties was evaluated using Human Umbilical Vein Endothelial Cells (HUVECs) subjected to in vitro cell migration scratch assays, tubule formation in Matrigel, cell viability and proliferation assays. In total, 25 different analogs were evaluated. Based on in vitro cell migration scratch assays, eight analogs were identified as potent angiogenesis inhibitors at 10 μM, a concentration that was determined to be nontoxic by colony formation assay. In addition, this approach identified a potent antiangiogenic ENOX1 inhibitor, analog **4b**.

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Endothelial cells form the lining of all the blood vessels and play a critical role in tissue growth and wound healing. They achieve this by retaining the ability to divide and proliferate in an adult vascular system by the process of 'angiogenesis', where new blood vessels are formed from existing cells via development of small capillaries.[1] Development of these capillaries, also known as neovascularization, plays a critical role in cell division, proliferation and movement.[1,2]

In the past half century research efforts have established a critical role for angiogenesis in tumor growth and metastasis.[2] Without development of a capillary network by neovascularization, a tumor will not have sufficient nutrients to grow beyond a limited size.[3] Thus, angiogenesis constitutes an important point in the control of cancer progression,[3] and consequently, several major cancer therapy advances that target angiogenesis have been made in the recent years. These include development of antiangiogenesis drugs such as bevacizumab that binds to vascular endothelial growth factor (VEGF), a pro-angiogenic growth factor, and inhibits VEGF–VEGF receptor interactions. Sunitinib and Sorafenib, small molecule receptor tyrosine kinase inhibitors, represent two other examples.[3–5] Other drugs that are currently in clinical trials include, AZD2171, a potent, indole-ether quinazoline that also targets all VEGF receptors in renal cell cancer.[6] While these treatments have been shown to be effective in controlling initial cancer progression, their effects have been shown to be limited due to adaptation or previously existing resistance.[6] This ability of tumor cells to adapt to current antiangiogenesis drugs underscores the need to develop novel drugs that not only target tumor vasculature but also complement cytotoxic therapy.[6,7]

We previously described the synthesis of novel compounds consisting of combinations of indole, benzyl, and quinuclidine moieties.[8–10] In these studies, the effect of three specific compounds on cellular proliferation and tubule formation ability of endothelial cells was analyzed in detail.[8] Both phenotypic screening and analysis of biochemical pathways affected by non toxic doses of the active compounds identified the ECTO-NOX (ENOX) family of cell surface enzymes as potential targets for these indole analogs.[8] The ENOX family of enzymes exhibit cell surface protein disulfide-thiol interchange activity and oxidize NAD(P)H as an alternate substrate.[8] Enox enzymes play a critical role in cell proliferation

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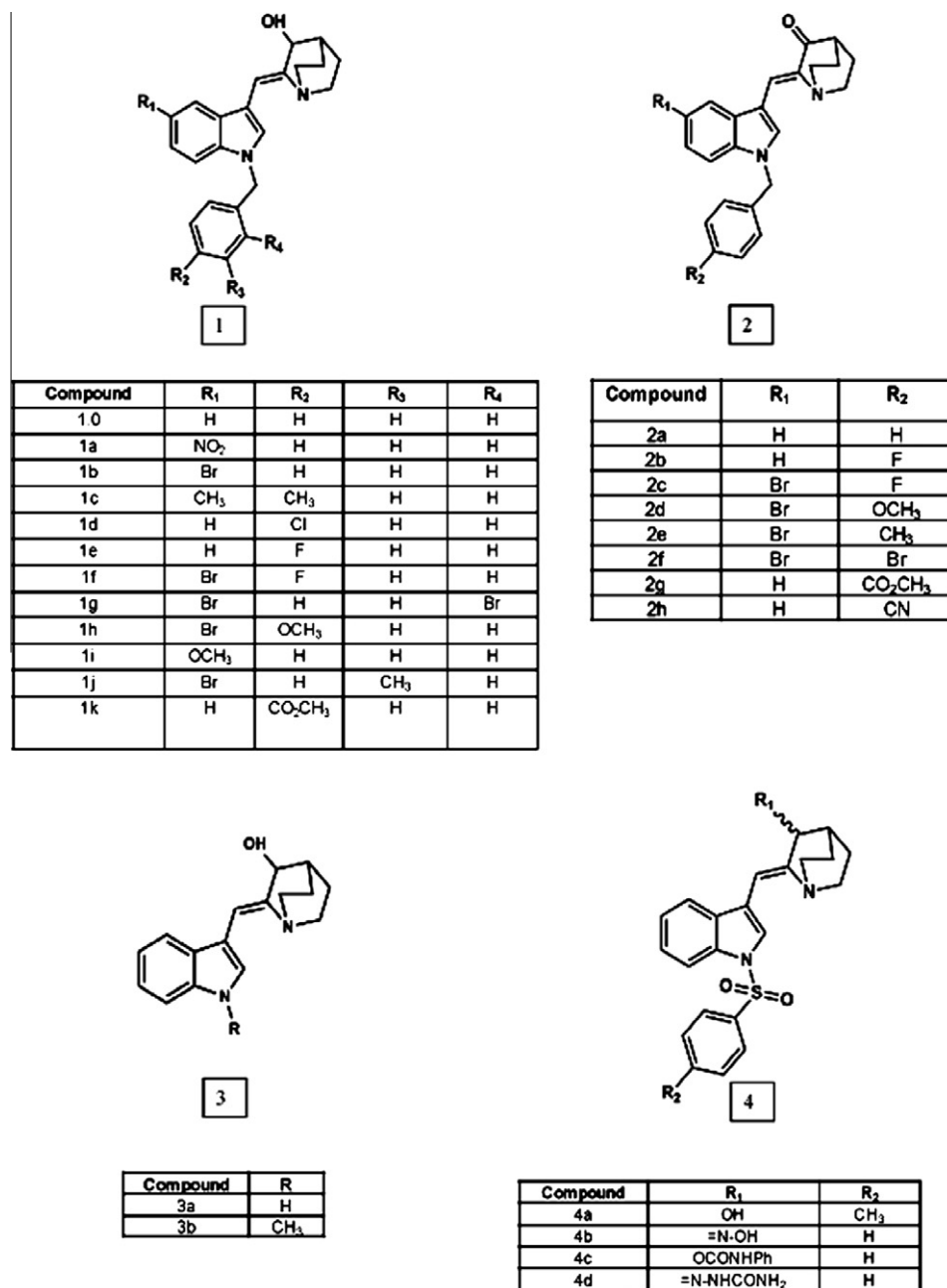E-mail address: raja.konjeti@Vanderbilt.Edu (K.R. Sekhar).
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and cancer.[11] The family consists of constitutively expressed ENOX1 (previously called CNOX), which is ubiquitous in mammals and plants, a cancer specific ENOX2 (formally called tNOX) that is thought to be unregulated and responsive to inhibitors, and an age-related ENOX.[12,13]

Our studies demonstrated that inhibition of ENOX activity by these indole compounds inhibited endothelial cell proliferation, inhibited the ability to form tubules, and synergistically increased radiation-mediated xenograft tumor growth delay. These observations led us to further investigate and establish the relationship between structure and antiangiogenesis activity of a series of (Z)-(±)-2-(N-benzylindol-3-ylmethylene)quinuclidin-3-ols (Fig. 1, **1a–1k**), (Z)-2-benzylindol-3-ylmethylene)quinuclidin-3-ones (**2a –2i**), (Z)-(±)-2-(1H/N-methyl-indol-3-ylmethylene)quinuclidin-3-ol (**3b**), and (Z)-(±)-2-(N-benzenesulfonylindol-3-yl-methylene)qui-nuclidin-3-ols and their derivatives (Fig. 1, **4a–4d**). In this study

we describe the effect of 25 additional derivatives on cellular migration of HUVECs. The synthesis of these analogs has been previously described.[10]

Initially, 25 novel analogs (Fig. 1) were analyzed for their ability to inhibit the migration of HUVECs in an in vitro cell migration scratch assay at a fixed concentration (25 μM). The scratch assay was performed as described in Geng et al.[8] HUVECs were seeded in 24-well culture plates. When confluent, a scratch was created using a 10 μl sterile pipette tip. Immediately following scratching, cells were washed twice with PBS to remove cell debris. Analogs were added at a final concentration of 25 μM in 1 ml final volume of HUVEC medium. Cells were incubated at 37 °C for 20 h. Microscopic images were taken at 0 h (immediately after drug addition) and at 20 h and the samples analyzed for cellular migration in the presence or absence of the test compound. Among analogs **1a–1k**, introduction of either halogen, methyl or methoxy substituents

Compound	R_1	R_2	R_3	R_4
1.0	H	H	H	H
1a	NO_2	H	H	H
1b	Br	H	H	H
1c	CH_3	CH_3	H	H
1d	H	Cl	H	H
1e	H	F	H	H
1f	Br	F	H	H
1g	Br	H	H	Br
1h	Br	OCH_3	H	H
1i	OCH_3	H	H	H
1j	Br	H	CH_3	H
1k	H	CO_2CH_3	H	H

Compound	R_1	R_2
2a	H	H
2b	H	F
2c	Br	F
2d	Br	OCH_3
2e	Br	CH_3
2f	Br	Br
2g	H	CO_2CH_3
2h	H	CN

Compound	R
3a	H
3b	CH_3

Compound	R_1	R_2
4a	OH	CH_3
4b	=N-OH	H
4c	OCONHPh	H
4d	=N-NHCONH$_2$	H

Figure 1. Chemical structures of novel quinuclidine compounds: structures of potent antiangiogenic agents with various substituents incorporated into the indole and *N*-benzyl moieties.
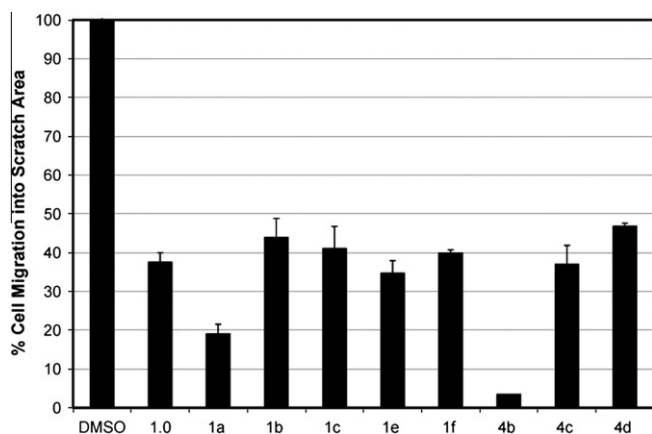
Figure 2. Scratch assay. The ability of HUVECs to migrate is inhibited by indole analogs. Eight of the 25 tested analogs were found to inhibit cellular migration of HUVECs at 25 µM concentration. Confluent endothelial cells in 24 well plates were scratched with a sterile 10 µl pipette tip. The cells were then allowed to grow in fresh medium with or without 25 µM of analog for 20 h.

Analogs that lacked aromatic substitution on the indole-NH were found to be inactive in migration assays (i.e., compounds **3a** and **3b**, Fig. 1).[8] Compound **3a** had been tested in our previous study,[8] and was used as negative control. Methyl substitution on the indole-NH (**3b**) did not improve the efficacy of compound **3a**. This observation suggests that benzyl substitution on the indole-NH is an important structural component that governs potency. Of the remaining analogs, eight compounds showed ≥50% decrease in HUVEC cell migration (Fig. 2). In our earlier study (Z)-(±)-2-(1-benzenesulfonylindol-3-ylmethylene)quinuclidin-3-ol was shown to exhibit potent antiangiogenic properties.[8] Since we have identified the OH group in quinuclidine moiety as an essential structural element, we modified this group to study the effect on cell migration. Among these modifications replacement of the OH group with an oxime group (**4b**) afforded a very potent compound (Fig. 2). Substitutions with semicarbazide and phenyl carbamate moieties yielded moderately active compounds. A total of eight of the 25 tested analogs (i.e., **1a–1c**, **1e**, **1f**, and **4b–4d**) were found to be potent inhibitors of cellular migration in HUVECs (Fig. 2) at 25 µM concentration. The ability to inhibit cell migration by these eight potent analogs was further tested at even lower concentrations (10 and 5 µM). All eight analogs were found to be effective cell migration inhibitors at 10 µM concentration (data not shown).

into the benzyl group did not yield a more potent compound than the unsubstituted analog (**1.0**) (Fig. 2). Introduction of electron withdrawing nitro group on the indole nucleus (**1a**) increased the potency of the parent compound (**1.0**). Interestingly, none of the (Z)-2-benzylindol-3-ylmethylene)quinuclidin-3-ones analogs (**2a–2i**) inhibit migration of HUVECs, suggesting that the OH group of the azabicyclo moiety is essential for inhibition of cell migration.

Based on results obtained from analysis of the scratch assay we chose the above eight analogs and tested their potency to inhibit the ability of HUVECs to form tubule-like structures. The assay was conducted in 24-well plates coated with Matrigel (BD Biosciences). HUVECs were plated onto the Matrigel in the presence or absence of the analogs (10 µM). Microscopic images were taken
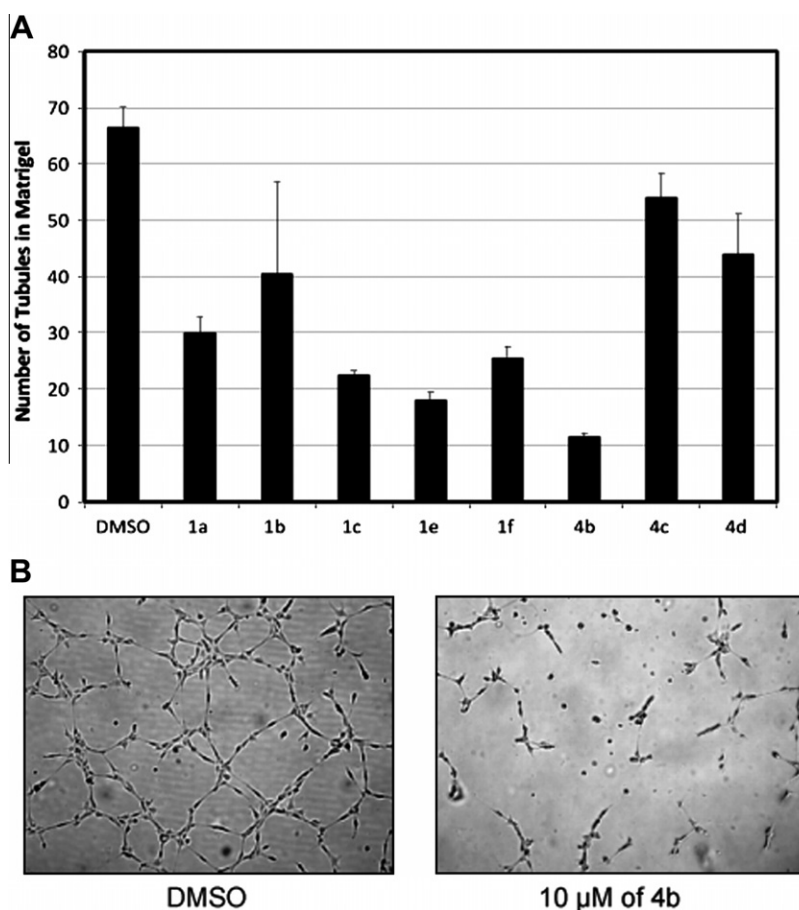
Figure 3. Tubule formation in Matrigel: the ability of HUVECs to form capillary-like structures is inhibited by analog **4b**. Endothelial cells were added to 24-well plates coated with Matrigel with or without 10 µM of analog **4b**. Plates were incubated for 7 h to allow formation of capillary-like structures. (A) Effect of various analogs on tubule formation ability of HUVECs. (B) The ability of HUVECs to form tubules in presence of analog **4b** (10 µM) was reduced by 83%.
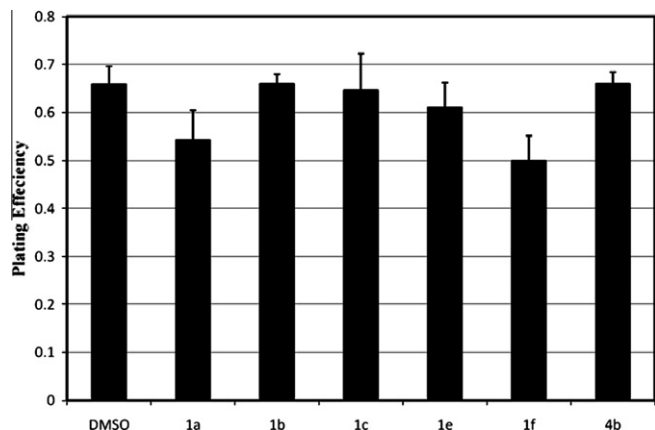
Figure 4. Colony formation assay: colony formation assays were used to assess the potency of six drug analogs.[9] HUVECs growing exponentially in T25 flasks (*n* = 4) were exposed to DMSO/analog for 6 h at 37 °C. Analog was then washed off, and cells were incubated in fresh medium for 14 days. Viable cells formed colonies that were stained with crystal violet and counted. The plating efficiency was defined as the ratio of the number of colonies counted divided by the initial number of cells exposed to vehicle control (DMSO) for 6 h. Vehicle treatment (6 h) was not toxic to cells, as survival was not significantly different from plating efficiency measured in the absence of vehicle (*p* >0.05, Student's *t*-test) and was arbitrarily set at 1.0. All analogs were tested at 10 μM. The percentage of surviving cells following exposure to analog alone was 75% or greater.
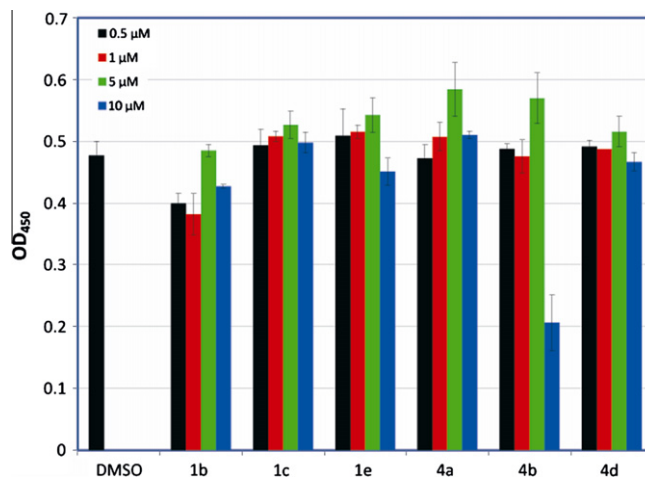
7 h after incubation, and the number of tubules formed was quantified by TotalLab software (Fig. 3). Consistent with our scratch assay, the tubule-like structure forming ability of HUVECs was suppressed by 83% in presence of analog **4b**.

A clonal plating efficiency assay[10] was used to assess the toxicity of the six most potent inhibitors (**1a**–**1c**, **1e**, **1f** and **4b**) identified from the two functional assays. These inhibitor analogs did not exhibit toxicity (Fig. 4), defined as a plating efficiency of less than 75% relative to the DMSO control, when tested at concentrations used in the tubule formation assay.

To determine if these analogs affected ECTO-NOX activity, we analyzed the reduction of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1)[8] in HUVECs in the presence of various concentrations of above six inhibitors. HUVECs in 100 μl of culture medium were seeded in 96-well plates and after 24 h of growth the cells were treated with analog (0.5, 1, 5, and 10 μM) or solvent (DMSO) in duplicate for 20 h. This was followed by a WST-1 assay, as per the manufacturer's (Calbiochem) recommendations. The optical density of each well was measured with a spectrophotometric microplate reader (Bio-Rad) at 450 nm. Interestingly none of the compounds inhibited WST-1 reduction, except for analog **4b** at 10 μM concentration (Fig. 5).

The goal of this study was to analyze the angiogenesis-inhibiting activity of novel indole-based analogs. From a pool of 25 different indole analogs, we identified eight analogs that had angiogenesis inhibition properties based on phenotypical assays: that is, cell migration and tubule formation in Matrigel. Compound **4b** was the most effective inhibitor and demonstrated an ability to inhibit ENOX activity, as determined by the WST-1 assay, as well as inhibition of phosphorylation of p70S6K1 at T389 (data not shown).[8] The kinase p70S6K1 has been previously shown to play a critical role in cell enlargement, proliferation and tubule formation.[14–16]

Indolone based compounds such as 3-substituted-2-oxoindole analogs, have been reported to possess antiangiogenic properties[16] independent of their serine/threonine kinase inhibition properties. Indolone analog SU5416, which inhibits VEGF2 activity, has been reported to possess antiangiogenic properties.[17] All these compounds lack a substituent on indole-NH. The striking difference between known antiangiogenic indole compounds and the compounds reported in this study is the presence of benzyl or benzene-sulfonyl substituent on the indole-NH. Lack of such a substituent renders the latter analogs inactive. Structural modifications with electron donating groups of either the indole moiety or the phenyl ring of the benzyl group on the indole-NH are not tolerated by these compounds with regard to their inhibition of cell migration and tubule formation. In conclusion, the present study has identified a potent ENOX inhibitor, **4b** that exhibits tyrosine kinase receptor-independent antiangiogenic properties.

Figure 5. Cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate): six potent analogs identified by scratch assay were evaluated for their ability to directly inhibit ECTO-NOX proteins by WST-1 assay at the indicated concentrations. Analog **4b** exhibited significant ENOX inhibition at 10 μM.

Acknowledgements

References and notes

1. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell*, 3rd ed.; Garland Publishing: New York, 1994.
2. *Cancer Medicine*; Kufe, D. W., Pollock, R. E., Weichselbaum, R. R., Bast, R. C., Jr., Gansler, T. S., Holland, J. F., Holland, J. F., Frei, Emil, III, Eds.; BC Decker: Hamilton, Canada, 2003.
3. Folkman, J. *Semin. Oncol.* **2002**, *29*, 15.
4. FDA Approval Summary for Bevacizumab—National Cancer Institute. November 10, 2008 http://www.cancer.gov/cancertopics/druginfo/fda-bevacizumab.
5. Mena, A. C.; Pulido, E. G.; Guillén-Ponce, C. *Anticancer Drugs* **2010**, *21*, 3.
6. Jeanny, B. A.; William, L. D. *Update Cancer Ther.* **2009**, *3*, 182.
7. Waldner, M. J.; Neurath, M. F. *Mol. Aspects Med.* **2010**, *31*, 171.
8. Geng, L.; Rachakonda, G.; Morré, D. J.; Morré, D. M.; Crooks, P. A.; Sonar, V. N.; Roti, J. L.; Rogers, B. E.; Greco, S.; Ye, F.; Salleng, K. J.; Sasi, S.; Freeman, M. L.; Sekhar, K. R. *FASEB J.* **2009**, *23*, 2986.
9. Sekhar, K. R.; Sonar, V. N.; Muthusamy, V.; Sasi, S.; Laszlo, A.; Sawani, J.; Horikoshi, N.; Higashikubo, R.; Bristow, R. G.; Borrelli, M. J.; Crooks, P. A.; Lepock, J. R.; Roti Roti, J. L.; Freeman, M. L. *Cancer Res.* **2007**, *67*, 695.
10. Reddy, Y. T.; Sekhar, K. R.; Sasi, N.; Reddy, P. N.; Freeman, M. L.; Crooks, P. A. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 600.
11. Morré, D. J.; Morré, D. M. *Free Radical Res.* **2003**, *37*, 795.
12. Tang, X.; Tian, Z.; Chueh, P. J.; Chen, S.; Morré, D. M.; Morré, D. J. *Biochemistry* **2007**, *46*, 12337.
13. Morré, D. J. *Plasma membrane Redox Systems and Their Role in Biological Stress and Disease.* Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998, p 121.
14. Montagne, J.; Stewart, M. J.; Stocker, H.; Hafen, E.; Kozma, S. C.; Thomas, G. *Science* **1999**, *285*, 2126.
15. Dufner, A.; Thomas, G. *Exp. Cell Res.* **1999**, *253*, 100.
16. Abadi, A. H.; Abou-Seri, S. M.; Abdel-Rahman, D. E.; Klein, C.; Lozach, O.; Meijer, L. *Eur. J. Med. Chem.* **2006**, *41*, 296.
17. Angelov, L.; Salhia, B.; Roncari, L.; McMahon, G.; Guha, A. *Cancer Res.* **1999**, *59*, 5536.