3,3-Diaryl-1,3-dihydroindol-2-ones as Antiproliferatives Mediated by Translation Initiation Inhibition

Amarnath Natarajan, Yun-Hua Fan, Han Chen, Yuhong Guo, Julia Iyasere, Frederick Harbinski, William J. Christ, Huseyin Aktas, and Jose A. Halperin*

Laboratory for Translational Research, Harvard Medical School, One Kendall Square, Building 600, 3rd Floor, Cambridge, Massachusetts 02139

Received January 8, 2004

Abstract: A series of substituted 3,3-diphenyl-1,3-dihydroindol-2-ones was synthesized from the corresponding isatins. The compounds were studied for cell growth inhibition mediated by partial depletion of intracellular Ca^{2+} stores that leads to phosphorylation of eIF2α. The diphenyloxindole (**1**) showed mechanism-specific antiproliferative activity that was comparable to known translation initiation inhibitors such as clotrimazole or troglitazone. SAR studies identified *m′-tert*-butyl and *o*-hydroxy substituted diphenyloxindole (**25**) as a lead compound for Ca^{2+}-depletion-mediated inhibition of translation initiation.

Introduction. Initiation of mRNA translation, a complex cellular process generally known as translation initiation, plays a critical role in the expression of oncogenic, prometastatic, and growth regulatory proteins.[1] Translation initiation is regulated by changes in the expression and/or phosphorylation status of the various translation initiation factors that configure the translation initiation machinery; for example, phosphorylation of eIF2α on its serine 51 residue reduces the overall rate of translation.[2] Translation initiation begins with the formation of a ternary complex between eIF2, GTP, and initiator methionine tRNA (met-tRNA). Phosphorylation of the α subunit of eIF2 prevents the formation of the eIF/GTP/met-tRNA complex, limits the rate of translation, and thereby inhibits protein synthesis.[3] We have demonstrated that clotrimazole (CLT) exerts a strong anticancer effect in cancer cells and tumors and that these anticancer properties are mediated by inhibition of translation initiation (Figure 1).[4] CLT exerts a distinct effect on the intracellular Ca^{2+} homeostasis; it releases Ca^{2+} from intracellular Ca^{2+} stores and at the same time inhibits restorative Ca^{2+} store-regulated Ca^{2+} influx through the plasma membrane. This results in a sustained partial depletion of the intracellular Ca^{2+} stores.[4,5] Depletion of intracellular Ca^{2+} stores activates eIF2 kinases (PKR and/or PERK) resulting in phosphorylation inactivation of eIF2α on serine 51. Inactivation of eIF2α primarily inhibits translation initiation resulting in the preferential down-regulation of oncogenes and growth-promoting proteins such as cyclin D1, ras, and c-myc.[6] More recently we have shown that Ca^{2+}-release-mediated inhibition of translation initiation also mediates the anticancer activity of other small molecules such as the n-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA)[7] and troglitazone (TRO),[8] hereon termed Ca^{2+}-depleting translation initiation inhibitors. Because Ca^{2+}-depleting translation initiation inhibitors preferentially modulate the synthesis of oncoproteins over "housekeeping" proteins, analogues of this type of compound have the potential to make robust anticancer drugs.

* To whom correspondence should be addressed. Phone: 617-621-6136. Fax: 617-621-6148. E-mail: jose_halperin@hms.harvard.edu.

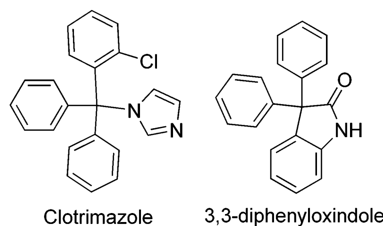
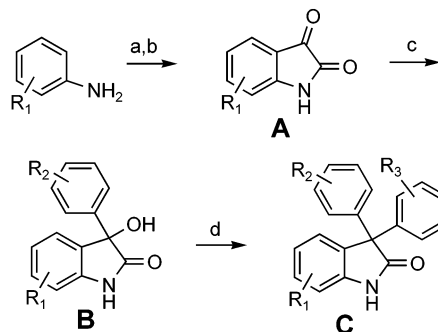


Figure 1. Clotrimazole and 3,3-diphenyloxindole Ca^{2+}-depleting translation initiation inhibitors.

Scheme 1. General Synthetic Approach to Diaryloxindole Analogues[a]



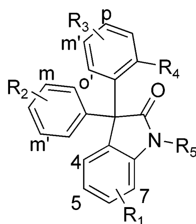
[a] (a) Chloral hydrate, $NH_2OH{\cdot}HCl$, HCl, Na_2SO_4, H_2O, reflux, 10 min; (b) concentrated H_2SO_4, heat, 1 h; (c) ArMgBr, 0 °C to room temperature; (d) ArR_3, *p*-TsOH, DCE, 85 °C or TfOH, DCM, room temperature.

As part of an ongoing project aimed at developing novel mechanism-specific anticancer agents, we recently identified 3,3-diphenyloxindole **1** (Figure 1) as a Ca^{2+}-depleting translation initiation inhibitor. In this letter, we report the synthesis of 3,3-diphenyloxindole analogues and their functional evaluation in translation initiation specific assays.

Chemistry. The general synthetic approach to the 3,3-diaryloxindole compounds is outlined in Scheme 1. The appropriate isatins **A** were either commercially available or synthesized starting from appropriate anilines.[10] As an example, **9** and **10** (Table 1) were synthesized starting from 3-bromoaniline, which upon condensation with hydroxylamine and chloral hydrate followed by concentrated sulfuric acid resulted in an inseparable regioisomeric mixture of 4- and 6-bromoisatins. The addition of the two phenyl groups to the 3-position of the oxindole allowed easy chromatographic separation of the 4- and 6-bromo-3,3-diphenyloxindoles **9** and **10**. A bioassay-guided iterative approach was undertaken in the study of the diaryloxindole compounds. The compounds synthesized addressed the electronics and sterics by substituting the three phenyl rings and the nitrogen with various functional groups. The symmetric diphenyl compounds were obtained using TfOH following previously reported procedures,[11]

10.1021/jm0499716 CCC: $27.50 © 2004 American Chemical Society
Published on Web 03/09/2004

Table 1. 3,3-Diaryloxindole Analogues[a] Assayed for Intracellular Ca^{2+} Depletion, eIF2α Phosphorylation, and Growth Inhibition



compd	R1	R2	R3	R4	R5	intracellular Ca^{2+} depletion	eIF2α phosphorylation[b]	GI_{50} (μM)
1	H	H	H	H	H	yes	1	13
2	5-F	H	H	H	H	yes	0.44	
3	5-Cl	H	H	H	H	yes	0.61	
4	5-Br	H	H	H	H	yes	0.79	
5	5-I	H	H	H	H	yes	1.31	8
6	5-Et	H	H	H	H	yes	0.85	>20
7	5-NO_2	H	H	H	H	yes	1.2	14
8	5-SO_3H	H	H	H	H	no		9
9	4-Br	H	H	H	H	yes	0.84	
10	6-Br	H	H	H	H	yes	0.89	
11	7-Br	H	H	H	H	no		
12	H	H	H	H	tosyl	no		
13	H	H	H	H	Ph	no		
14	H	H	H	H	Me	no		>20
15	H	*o*-OMe	H	H	H	yes	0.48	
16	H	*m*-OMe	H	H	H	no		
17	H	*p*-OMe	H	H	H	no		
18	H	*o*-OH	H	H	H	yes	0.56	
19	H	*p*-OH	H	H	H	yes	0.86	
20	H	*m*-*t*-Bu	H	H	H	yes	1.04	4
21	H	*p*-*t*-Bu	H	H	H	yes	0.95	8
22	H	*o*-OMe	*p*-*t*-Bu	H	H	no	0.93	
23	H	*o*-OH	*p*-*t*-Bu	H	H	yes	1.33	7
24	H	*p*-OH	*p*-*t*-Bu	H	H	yes	2.01	14
25	H	H	*m'*-*t*-Bu	OH	H	yes	2	3
26	5-I	H	*m'*-*t*-Bu	OH	H	yes	1.4	4
27	H	H	*m'*-CF_3	OH	H	no		>20

[a] All compounds were characterized and purity was assessed using ^1H NMR and LCMS. [b] Normalized to **1**.

while the unsymmetric compounds were synthesized using a two-step procedure. The first step involved a Grignard addition to isatin using appropriately substituted arylmagnesium halide, and the key second step involved the generation of a quaternary center at the 3-position of the oxindole ring.[12] This was accomplished by an acid-catalyzed Friedel−Crafts type condensation of **B** (Scheme 1) with the appropriate aromatic ring to generate diaryloxindoles **C**. For activated ring systems that contained an electron-donating group, *p*-toluenesulfonic acid in dichloroethane allowed the condensation to occur smoothly, while the unactivated ring systems required the use of triflic acid (a much stronger acid).[13]

Biology. All compounds synthesized were first screened for their ability to release Ca^{2+} from internal stores using FURA-2AM loaded cells assayed in Ca^{2+} free media, as previously described.[6−8] Compounds that release Ca^{2+} in this assay were further evaluated for their ability to phosphorylate eIF2α and were quantified by Western blot analysis using both a phospho-specific and a total anti-eIF2α antibody.[9] The compounds that released intracellular Ca^{2+} and phosphorylated eIF2α were further tested for anticancer activity in vitro using a human lung cancer cell line, as previously described.[6−8]

The endoplasmic reticulum (ER) is a main site of cellular Ca^{2+} storage. To formally demonstrate that the 3,3-diaryloxindoles compounds partially deplete the ER-calcium stores, we tested our lead compound, **25**, in an ER-Ca^{2+} specific assay that uses stable cells lines carrying ER-targeted Ca^{2+}-sensitive cameleon proteins.[14] These proteins emit light, and the Ca^{2+} content is measured by fluorescent resonance energy transfer (FRET), which is a function of the Ca^{2+} content of the ER.

Results and Discussion. To develop Ca^{2+}-depleting translation initiation inhibitors, we took a bioassay-guided iterative approach. Ca^{2+}-depleting translation initiation inhibitors are those compounds that induce Ca^{2+} release from intracellular stores, phosphorylation of eIF2α, and growth inhibition, as summarized in Table 1.

Several 5-substituted isatins are commercially available; hence, the first batch of compounds involved the variation of the functional groups at the 5-position on the oxindole phenyl ring (**2**−**8**) to explore the size and electronic effects of the substituents on the biological activity. Interestingly, all the substitutions except a sulfonic acid group generated compounds that released intracellular Ca^{2+}. Given the short time course of our Ca^{2+} release assay (minutes), the absence of Ca^{2+} depletion activity in the sulfonic acid analogue could be due in part to poor cell permeability. The apparently high growth inhibitory effect of **8** may reflect increased cellular toxicity. Comparison of eIF2α phosphorylation among **1**−**8** showed a good correlation between the size of the substituent at the 5-position and the eIF2α phosphorylation activity of the compounds. The 5-iodo substitution increased eIF2α phosphorylation by 20%

compared to the parent compound **1** and also exhibited stronger inhibitory activity on cancer cells growth. In contrast, an ethyl substitution at the 5-position of the oxindole ring (**6**) resulted in a 15% reduction in eIF2α phosphorylation, which was also reflected in a reduced inhibition of cancer cells growth.

The positional effect (4, 5, 6, and 7) of a substituent on the phenyl ring of oxindole on the biological activity was investigated by synthesizing **9**, **4**, **10**, and **11** with a bromo group at positions 4, 5, 6, and 7, respectively. The 7-Br substitution in **11** abolished the Ca^{2+} depletion activity, and the other substitutions did not result in any improvement of the biological activities. Interestingly, substituting the nitrogen (**12**−**14**) with alkyl, aryl, and electron-withdrawing groups abolished the Ca^{2+} depletion activity.

We then turned our attention to substituting the phenyl rings at the 3-position of the oxindole ring. We placed electron-releasing groups such as methoxy, hydroxy, and *tert*-butyl at different positions on one of the phenyl rings (**15**−**21**). We observed a loss of Ca^{2+} depletion activity with meta- and para-substituted methoxy groups (**16** and **17**). However, the ortho-substituted methoxy, hydroxyl (**15**, **18**), and the para-substituted hydroxyl showed Ca^{2+} depletion but with diminished eIF2α phosphorylation activity compared to **1**. It is interesting to note that both methoxy and hydroxyl substitutions at the ortho position (**15** and **18**) showed Ca^{2+} depletion but not the para-substituted methoxy compound (**17**); this result suggests that the biological activity of these compounds is mediated by a nonelectronic effect. Most rewarding in this monosubstituted series was the *t*-Bu substitution at meta and para positions, (**20** and **21**, respectively), which caused Ca^{2+} depletion and eIF2α phosphorylation with improved growth inhibitory activity. These results prompted us to differentially substitute the two phenyl rings (**22**−**24**). Although, the *m-t*-Bu substituted compound **20** shows better activity compared to the *p-t*-Bu substituted compound **21**, we decided to use the *p-t*-Bu for the differential substitution because the *p-t*-BuPhMgBr is commercially available. As predicted, ortho and para hydroxyl substitutions on one ring and a para *tert*-butyl on the other (**23** and **24**) resulted in a significant improvement of biological activity compared to the parent compound **1**. Interestingly, the *o*-OMe substitution (**22**) resulted in an inactive compound.

Structure−function analysis of **18**−**24** led us to speculate that placing the *t*-Bu and the hydroxy on one phenyl ring might result in a better Ca^{2+}-depleting translation initiation inhibitor. To test this hypothesis, we synthesized and assayed **25**, which has an *o*-hydroxy and a *m′-t*-Bu on the same ring.[15] This compound showed very good Ca^{2+}-depleting activity, a 2-fold increase in the eIF2α phosphorylation, and a 4-fold improvement in the growth inhibition assay. Furthermore, **25** induced a depletion of ER-Ca^{2+}, as determined in cells carrying ER targeted Ca^{2+}-sensitive cameleon protein. Combined, these results indicate that the growth inhibitory activity of **25** is most likely mediated by partial depletion of ER-Ca^{2+} stores, phosphorylation of eIF2α, and its downstream effects on cell cycle regulatory proteins.

Since the 5-iodo substituted compound **5** was the most active compound among the monosubstituted series, we synthesized **26** to explore the effect of combining a hydroxy at the ortho position and a *t*-Bu at the meta′ position of the 3-phenyl ring of **25**, with the iodo group at the 5-position of the phenyl ring of the oxindole, as in **5**. Contrary to our expectation, these combined substitutions did not improve the biological activity. Interestingly, replacing the *t*-Bu on **25** with a trifluoromethyl group (**27**) completely abolished the Ca^{2+}-depleting and the growth inhibitory activity observed in the parent compounds.

In conclusion, we have identified 3,3-diaryloxindoles as Ca^{2+}-depleting translation initiation inhibitors. Using a bioassay-guided modification approach, we have identified a new mechanism-specific lead, **25**, which induced partial depletion of ER-Ca^{2+} stores, is more potent than its parent compound **1** in inducing eIF2α phosphorylation, and is also 4 times more potent in inhibiting cancer cells growth. We are currently using **25** as a lead compound in our efforts to identify the molecular target for ER Ca^{2+} depletion. We are also conducting experiments to evaluate **25** preclinically in animal models of experimental cancer.

Acknowledgment. We thank Drs. Daniel C. Tosteson, Magdalena Tosteson, and Michael Chorev for comments and continuous support. This work was supported in part by NIH Grant NCDDG 5 U19 CA 87427.

References

(1) (a) Kozak, M. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **1991**, *115*, 887−903. (b) Kozak, M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **1991**, *266*, 19867−19874.

(2) Meric, F.; Hunt, K. K. Translation initiation in cancer: A novel target for therapy. *Mol. Cancer Ther.* **2002**, *1*, 971−982.

(3) (a) Brostrom, C. O.; Brostrom, M. A. Regulation of translational initiation during cellular responses to stress. *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *58*, 79−125. (b) Pain, V. M. Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* **1998**, *236*, 747−771.

(4) Benzaquen, L. R.; Brugnara, C.; Byers, H. R.; Gattoni-Celli, S.; Halperin, J. A. Clotrimazole inhibits cell proliferation in vitro and in vivo. *Nat. Med.* **1995**, *1*, 534−540.

(5) Brostrom, C. O.; Prostko, C. R.; Kaufman, R. J.; Brostrom, M. A. Inhibition of translational initiation in eukaryotic cells by calcium ionophore. *J. Biol. Chem.* **1996**, *271*, 24995−25002.

(6) Aktas, H.; Fluckiger, R.; Acosta, J. A.; Savage, J. M.; Palakurthi, S. S.; Halperin, J. A. Depletion of intracellular Ca^{2+} stores, phosphorylation of eIF2α, and sustained inhibition of translation initiation mediate the anticancer effects of clotrimazole. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8280−8285.

(7) Palakurthi, S. S.; Fluckiger, R.; Aktas, H.; Changolkar, A. K.; Shahsafaei, A.; Harneit, S.; Kilic, E.; Halperin, J. A. Inhibition of translation initiation mediates the anticancer effect of the n-3 polyunsaturated fatty acid eicosapentaenoic acid. *Cancer Res.* **2000**, *60*, 2919−2925.

(8) Palakurthi, S. S.; Aktas, H.; Gubissich, L. M.; Mortensen, R. M.; Halperin, J. A. Anticancer effects of thiazolidinedione's are independent of peroxidome proliferator-activated receptor γ and mediated by inhibition of translation initiation. *Cancer Res.* **2001**, *61*, 6213−6218.

(9) Aktas, H.; Cai, H.; Cooper, G. M. Ras links growth factor signalling to the cell cycle machinery via regulation of cyclin D1 and the cdk inhibitor $p27^{kip1}$. *Mol. Cell. Biol.* **1997**, *17*, 3850−3857.

(10) (a) Deady, L. W.; Kaye, A. J.; Finlay, G. J.; Baguley, B. C.; Denny, W. A. Synthesis and Antitumor Properties of *N*-[2-(Dimethylamino)ethyl]carboxamide Derivatives of Fused Tetracyclic Quinolines and Quinoxalines: A New Class of Putative Topoisomerase Inhibitors. *J. Med. Chem.* **1997**, *40*, 2040−2046. (b) Ma, D.; Wu, Q. Enantioselective construction of the oxidized tryptophan fragment of proteasome inhibitors TMC-95A and TMC-95B. *Tetrahedron Lett.* **2000**, *41*, 9089−9093.

(11) Klumpp, A. A.; Yeung, K. Y.; Surya Prakash, G. K.; Olah, G. A. Preparation of 3,3-Diaryloxindoles by Superacid-Induced Condensations of Isatins and Aromatics with a Combinatorial Approach. *J. Org. Chem.* **1998**, *63*, 4481−4484.

(12) Hewawasam, P.; Gribkoff, V. K.; Pendri, Y.; Dworetzky, S. I.; Meanwell, N. a.; Martinez, E.; Boissard, C. G.; Post-Munson, D. J.; Trojnacki, J. T.; Yeleswaram, K.; Pajor, L. M.; Knipe, J.; Gao, Q.; Perrone, R.; Starrett, J. E., Jr. The Synthesis and Characterization of BMS-204352 (MaxiPost) and Related 3-Fluorooxindoles as Openers of Maxi-K Potassium Channels. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1023−1026.

(13) Nicolaou, K. C.; Bella, M.; Chen, D. Y.-K.; Huang, X.; Ling, T.; Snyder, S. A. Total Synthesis of Diazonamide A. *Angew. Chem., Int. Ed.* **2002**, *41*, 3495−3499.

(14) Miyawaki, A.; Mizuno, H.; Llopis, J.; Tsien, R. Y.; Jalink, K. *Cameleons as Cytosolic and Intra-Organellar Calcium Probes*; Oxford University Press: Oxford, U.K., 2001; pp 3−16.

(15) (5-*tert*-Butyl-2-hydroxyphenyl)-3-phenyl-1,3-dihydroindol-2-one (**25**): To isatin (15 g, 0.1 mole) in 60 mL THF at 0 °C was added dropwise a 1 M solution of phenylmagnesium bromide (0.33 mol). The resulting mixture was allowed to warm to room temperature and was spun at room temperature for 12 h. The reaction was quenched with a saturated solution of NH_4Cl (100 mL), and the mixture was diluted with 100 mL of dichloromethane. The layers were separated, and the organic layer was washed with water followed by brine, dried over Na_2SO_4, and filtered. The filterate was concentrated in vacuo to yield 3-hydroxy-3-phenyl-1,3-dihydroindol-2-one (20.1 g, 90%) as a pale-yellow solid. A mixture of the above solid (2.5 g, 10 mmol), *p*-*t*-Bu-phenol (1.5 g, 10 mmol) and *p*-toluenesulfonic acid (3 g) in 40 mL of dichloroethane, was heated to 95 °C for 6 h. The mixture was cooled and filtered, and the filterate was concentrated in vacuo. Flash chromatography of the resulting solid yielded **25** (2.4 g, 66%) as a colorless solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 9.24 (s, 1H), 7.23−7.34 (m, 5H), 7.18 (t, $J = 8.0$ Hz, 1H), 7.08 (dd, $J = 8.0, 1.6$ Hz, 1H), 7.01 (d, $J = 7.2$ Hz, 1H), 6.93 (t, $J = 8.0$ Hz, 1H), 6.86 (d, $J = 7.2$ Hz, 1H), 6.74 (d, $J = 1.6$ Hz, 1H), 6.59 (m, 1H), 1.07 (s, 9H); ^{13}CNMR (125 MHz, DMSO-d_6) δ 180.22, 153.56, 143.14, 140.92, 140.28, 133.49, 129.08, 128.94, 128.60, 128.47, 127.88, 127.42, 126.30, 125.43, 121.79, 115.73, 110.01, 60.70, 34.36, 31.99 ppm; MS (APCI$^+$) m/z 358.23 (MH), 302.18 (loss of *t*-Bu), 208.14 (loss of *tert*-butylphenol).

JM0499716