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Letters

3,6-Dibromocarbazole Piperazine Derivatives of 2-Propanol as First Inhibitors of Cytochrome *c* Release via Bax Channel Modulation

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Abstract: There is compelling evidence that Bax channel activity stimulates cytochrome *c* release leading ultimately to cell death, which is a key event in ischemic injuries and neurodegenerative diseases. Here 3,6-dibromocarbazole piperazine derivatives of 2-propanol are described as the first small and potent modulators of the cytochrome *c* release triggered by Bid-induced Bax activation in a mitochondrial assay. Furthermore, a mechanism of action is proposed, and fluorescent derivatives allowing the localization of such inhibitors are reported.

Introduction. Several pathologies, including ischemic injuries and neurodegenerative diseases, are associated with inappropriate cell death. During disease, cells can die by either necrosis or apoptosis. In contrast to necrosis, apoptosis is a highly controlled and regulated process.[1] The mitochondrial apoptosis pathway is controlled by the Bcl-2 family of proteins, which comprises both anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-ω, Mcl-1, A1) and pro-apoptotic (Bax, Bak, Bok/Mtd, BCl-Xs, Bid, Bad, Bik/Nbk, Bim, Blk) proteins.[2] The principal site of action of the Bcl-2 family members is the mitochondria. When activated during apoptosis, multidomain pro-apoptotic proteins, such as Bax and Bak, permeabilize the outer mitochondrial membrane, triggering the release of several proteins, including cytochrome *c*, from the intermembrane space.[3] In the cytosol, cytochrome *c* forms a complex with Apaf-1, dATP, and procaspase-9, which leads to caspase 9 activation followed by downstream activation of other caspases, ultimately leading to cell death.[4] BH3 domain only pro-apoptotic proteins, such as Bid, work upstream of the multidomain proteins promoting their activation and cytochrome *c* releasing activity.[5] After caspase 8 cleavage, the 15.5 kDa C-terminal fragment of Bid interacts with Bax and Bak, inducing conformational changes in these proteins, which lead to their oligomerization and activation.[6] In contrast, anti-apoptotic family members, such as Bcl-2 and Bcl-xL, have been shown to inhibit Bax and Bak activation, suppressing cytochrome *c* release.

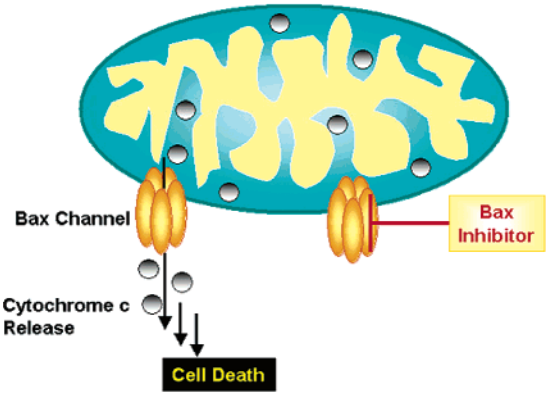

Figure 1. Model of the mitochondrial apoptosis pathway. Bax induces release of cytochrome *c* from mitochondria presumably through formation of channels. A Bax channel inhibitor preventing cytochrome *c* release may protect against cellular death.

There is compelling evidence from both in vitro studies using isolated mitochondria as well as in intact cells following heterologous expression that Bax channel activity stimulates cytochrome *c* release. A Bax channel inhibitor would be expected to prevent cytochrome *c* release from mitochondria during apoptosis, which is viewed to be useful to protect neurons and other cell types from various cell death stimuli (Figure 1). Very few small molecule inhibitors of the Bcl-2 family have been described so far, among them tricyclic derivatives as inhibitors of Bcl-2 itself.[7] Subsequent substructure

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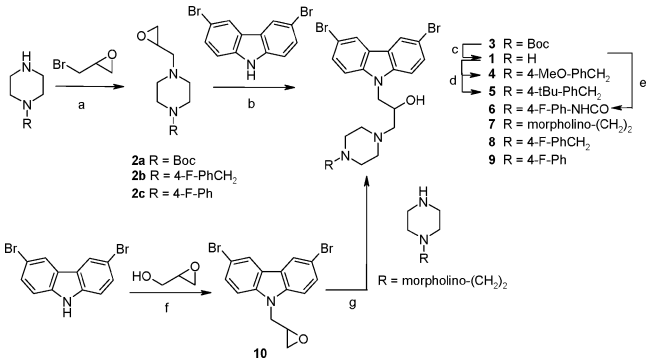
Scheme 1[a]

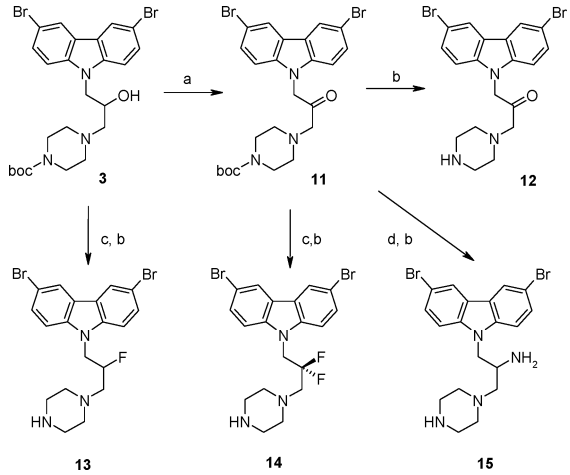


[a] Reagents: (a) K_2CO_3, ACN, room temp, 80−100%; (b) NaH, THF, 60 °C, 40−80%; (c) TFA, DCM, room temp, 80%; (d) 4-MeO-PhCHO or 4-*tert*-Bu-PhCHO, $NaBH(OAc)_3$, TMOF, room temp, 43% or 48%, respectively; (e) 4-F-PhNCO, $^iPr_2EtN$, DCM, room temp, 48%; (f) DEAD, PPh_3, THF, room temp, 65%; (g) EtOH, reflux, 80−100%.

analogy search led us to consider carbazole derivatives as potential starting points. This hypothesis was validated by first evaluating the commercially available 3,6-dibromocarbazole derivative **1** which turned out to inhibit cytochrome *c* release from mitochondria via Bax channel modulation. In this paper we describe compound **1** and 3,6-dibromocarbazole piperazine derivatives of 2-propanol as the first small and potent modulators of the cytochrome *c* release triggered by Bid-induced Bax activation in a mitochondrial assay.

Chemistry. The chemistry to prepare 1-(3,6-dibromocarbazol-9-yl)-3-piperazin-1-yl-propan-2-ol **1** and analogues is depicted in Scheme 1. Synthesis was used to assemble the three fragments: the piperazine moiety, the propanol spacer and the 3,6-dibromocarbazole ring. Treatment of (±)-epibromohydrin with the appropriate substituted piperazine in the presence of potassium carbonate in acetonitrile (ACN) gave the piperazine epoxide compounds **2** in high yield (>80%). Ring opening of the epoxide moiety by a solution of deprotonated 3,6-dibromocarbazole in anhydrous tetrahydrofuran (THF) at 60 °C gave the desired 1,3-disubstituted-2-propanol derivatives in moderate to good yields (40−80%, compounds **3**, **8**, and **9**). The protecting group *tert*-butyloxycarbonyl (Boc) of compound **3** was conveniently removed by the use of trifluoroacetic acid (TFA) in a dichloromethane (DCM) solution and gave the corresponding free amine **1** in 80% yield (Scheme 2). The benzylic piperazine derivatives, e.g., **4** and **5**, were obtained by reductive alkylation with the appropriate benzaldehydes in the presence of triacetoxy borohydride in trimethyl orthoformate (TMOF) with moderate yields (40−50%). Exposure of compound **1** to 4-fluorophenyl isocyanate gave the urea compound **6** in 48% yield. In parallel, an improved one-step synthesis of 3,6-dibromocarbazole piperazine derivatives of 2-propanol was developed, starting from commercially available substituted piperazines and 3,6-dibromo-9*H*-(oxiran-2-ylmethyl)carbazole **10**. Intermediate **10** was prepared from 3,6-dibromocarbazole and (±)-glycidol under Mitsunobu reaction conditions (diethylazodicarboxylate (DEAD)/triphenylphosphine (TPP) in THF) in 65% yield.[8] Reaction of **10** with appropriately substituted piperazines in ethanol at reflux led to desired final compounds in excellent yield (e.g., compound **7**, 97%).

Scheme 2. Modification of the Propanol Spacer[a]



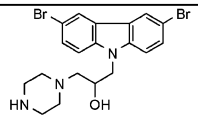
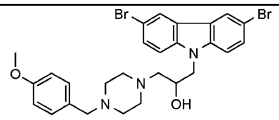
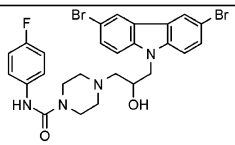
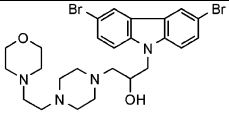
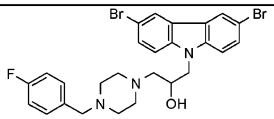
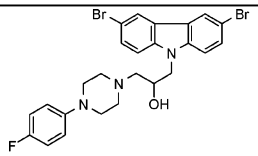
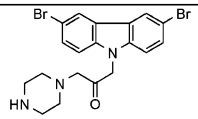
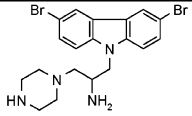
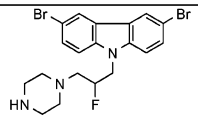
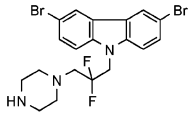
[a] Reagents: (a) $(COCl)_2$, DMSO, Et_3N, DCM, −78 to −30 °C, 81%; (b) TFA, DCM, room temp, 80−100%; (c) DAST, DCM, 0 °C, 20−40%; (d) i. NH_3, TMOF, 60 °C; ii. $NaBH_4$, MeOH, room temp, 58%.

Compounds, featuring substitution of the propyl linker, were prepared using intermediate **3** as starting material, following the synthetic sequence described in Scheme 2. Swern oxidation of compound **3** gave the ketone **11**, which was converted to the corresponding free amine **12** after Boc removal with TFA in an overall yield of 67%. The mono and difluoro compounds **13** and **14** were obtained from the reaction of compounds **3** and **11**, respectively, using (diethylamino)sulfur trifluoride (DAST) in DCM at 0 °C and a further exposure to TFA for Boc removal in overall yields of 40 and 20%, respectively. Reductive amination of ketone **11** by treatment with ammonia in TMOF and reduction with sodium borohydride in the presence of methanol gave the amino derivative **15**, after Boc removal with an overall yield of 58%.

Results and Discussion. The synthesized compounds were evaluated for their in vitro inhibitory activity of cytochrome *c* release triggered by Bid-induced Bax activation in isolated mitochondria as described by Antonsson et al.[9] The activity of the 3,6-dibromocarbazole piperazine derivatives of 2-propanol was assayed at 10 μM, and cytochrome *c* release was monitored by Western blotting. Table 1 summarizes the data for representative compounds. Initially, the investigation of the SAR around compound **1** focused on the propanol spacer by introducing diverse substituents on the propyl chain, while keeping the piperazine moiety constant, since these modifications are synthetically readily accessible. Replacement of the hydroxy group by a pure hydrogen bond acceptor (keto, compound **12**) or donor (amino, compound **15**), as well as monofluoro (compound **13**) and difluoro groups (compound **14**), resulted in a decrease of activity (see Table 1). The monohydroxy analogue **1** remains the unprecedented quasi-total inhibitor of Bax-induced cytochrome *c* release at 10 μM. Both enantiomers of compound **1** were synthesized in a pure form, tested, and identified as equipotent inhibitors of cytochrome *c* release.[10]

The high potency observed for the substituted propyl analogues encouraged us to extend the SAR exploration to the *N*-piperazine moiety. Preliminary biological evaluation of intermediates such as the *N*-Boc derivatives **3**

Table 1. In Vitro Inhibition of Cytochrome *c* Release in Isolated Mitochondria and IC_{50} in Bax Channel Liposome Assays

Compd	Structure	Inh (%) of Cyt c Release*	IC50 (μM) Bax assay
1		99±2 (20 μM) 94±4 (10 μM) 77±7 (5 μM)	0.52 ±0.21
4		99±2 (20 μM) 82±5 (10 μM) 61±5 (5 μM)	0.37 ±0.12
6		15±5	1.27 ±0.37
7		95±2 (20 μM) 74±9 (10 μM) 21±10 (5 μM)	0.47 ±0.07
8		65±6	0.48 ±0.14
9		15±10	2.38 ±0.48
12		71±5	0.29 ±0.04
15		71±10	0.96 ±0.10
13		97±3 (20 μM) 83±11 (10 μM) 40±9 (5 μM)	0.68 ±0.22
14		96±3 (20 μM) 83±10 (10 μM) 61±2 (5 μM)	0.68 ±0.09

*Inhibition of cytochrome *c* release triggered by Bid-induced Bax activation using 10 μM of compounds unless specified differently. Values are the mean of two or three experiments in duplicate ± SD.

and **11** showed us that a dramatic reduction of activity was observed with carbamate substitution on the piperazine (not shown). This observation was confirmed with the fluoro aryl urea **6**, which was also found inactive in this assay. These preliminary data suggested the importance of the piperazine basic nitrogen. Introduction of an aliphatic group on the piperazine showed good activity. For instance, the morpholino analogue **7** showed 74% inhibitory activity. In general, aromatic substitutions, especially bearing small substituents with little lipophilic and electronic effects demonstrated similar activity. Thus 4-methoxy and 4-fluoro benzyl analogues **4** and **8** exhibited 82 and 65% inhibition, respectively. It was noteworthy that aromatic substituents with large molar refractivity, e.g., *tert*-butyl of analogue **5**, were detrimental (data not shown). The spacer between the aromatic substitution and the piperazine was essential, which underlined the role of the basic piperazine nitrogen: compounds with a urea **6** or no spacer **9** showed no significant activity at 10 μM. Taking into account the SAR optimization of the piperazine ring, a few substitutions were tried with the fluoro propyl spacer. No additive effect was observed, presumably because of too high lipophilicity leading to low solubility of the final compounds.

Furthermore, five representative compounds were evaluated at two additional concentrations (20 and 5 μM) and showed a dose dependent inhibition of cytochrome *c* release as illustrated in Table 1. These compounds inhibited the cytochrome *c* release totally at 20 μM. At 5 μM, compound **1** showed the most potent inhibition (77 ± 7%), whereas weaker inhibition was observed for compounds **4** and **13**, and almost no inhibition for compound **7**.

Although it is not fully understood how Bax regulates the release of cytochrome *c*, one suggested mechanism is that formation of channels are critical for its biological function. Using an in vitro liposome channel assay,[11] Bax modulation of compounds was evaluated. This test is based on the ability of compounds to block the channel activity in a liposome assay where liposomes are charged with the fluorescent dye carboxyfluorescein. Addition of compounds to the assay solution inhibited Bax channel-forming activity in a concentration dependent manner as illustrated in Table 1. The IC_{50} value of compound **1** was determined to be 0.52 ± 0.21 μM, suggesting that compound **1** is a Bax channel blocker as hypothesized. In addition, compounds showing significant inhibition (>65%) of cytochrome *c* release at 10 μM also show sub-micromolar IC_{50} value in the liposome assay, while compounds showing poor activity on cytochrome *c* release (compounds **6** and **9**) are weak inhibitors of Bax channel activity. These results are in agreement with the hypothesis that the inhibition of cytochrome *c* release can indeed be mediated and correlated by the ability of compounds to inhibit Bax channel activity. Other possible mechanisms of action such as inhibition of Bax insertion into the lipid membrane and/or prevention of Bax channel assembly are currently under investigation and will be published in a subsequent article.

To investigate the intracellular localization of the possible site of action of the compounds, we introduced a fluorescent moiety into the identified inhibitors of cytochrome *c* release. Knowing the previously described SAR of the series, compound **1** was modified to introduce a fluorescent moiety at the piperazine nitrogen. Different fluorescent dyes such as BODIPY (using BODIPY 493/503 methyl bromide) and fluorescein (using 5-(iodoacetamido)fluorescein (5-IAF)) were evaluated resulting in the design of compounds **16** and **17**, whose synthesis is outlined in Scheme 3.

Fluorescent compounds showed acceptable in vitro activity (50% inhibition of cytochrome *c* release triggered

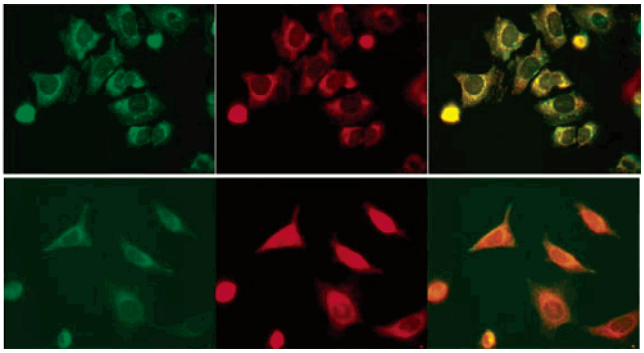
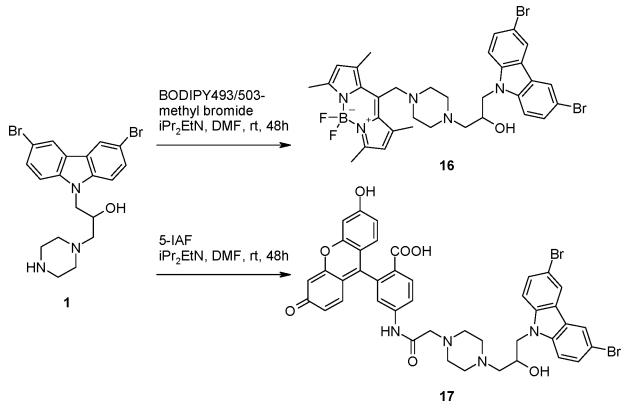


Figure 2. Localization of compounds **16** (upper) and **17** (lower) in HeLa Cells. On the left, cells were incubated with 2 μM of inhibitors **16** or **17** and 0.1 μM of mitotraker (middle) at 37 °C for 1 h. **16** and **17** are colocalized with mitotraker as seen by overlaying it the green and the red images (right), suggesting their site of action as the mitochondria.

Scheme 3. Synthesis of Fluorescent Derivatives



by Bid-induced Bax activation at 10 μM) and were used as a pharmacological tool to study the location of modulators of Bax as shown in Figure 2. The introduction of the fluorescent moiety allowed determining the localization of the compounds after incubation with HeLa cells. The compounds were found to be colocalized with the mitochondrial marker Mitotraker (Figure 2, middle pictures). This suggests that 3,6-dibromocarbazole piperazine derivatives of 2-propanol modulate the release of cytochrome *c* in vitro by interaction with some mitochondria components.

In conclusion, here we described for the first time small molecules as modulators of cytochrome *c* release, which is a key event in the mitochondrial apoptosis pathway. Compound **1** and a series of its derivatives were synthesized and evaluated for their ability to inhibit the release of cytochrome *c* from mitochondria. Preliminary studies strongly suggest that compound **1** is able to interact with Bax and this presumably at the mitochondria. Notwithstanding the therapeutic potential of these new anti-apoptotic compounds, they can be used as a research tool to dissect the functioning of the apoptotic cascades.

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Supporting Information Available: Detailed chemistry and biological experimental procedures for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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