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Adamantyl-tethered-biphenylic compounds induce apoptosis in cancer cells by targeting Bcl homologs

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ABSTRACT

Bcl homologs prominently contribute to apoptotic resistance in cancer cells and serve as molecular targets in treatment of various cancers. Herein, we report the synthesis of biphenyl-adamantane derivatives by a ligand free palladium on carbon based Suzuki reaction using diisopropylamine as a base for the coupling of adamantane based aryl chloride with a variety of aryl boronic acids. Among the biphenyl derivatives synthesized, compound 3′-(adamantan-1-yl)-4′-methoxy-[1,1′-biphenyl]-3-ol (AMB) displayed cytotoxic activity against hepatocellular carcinoma cell lines without significantly affecting the normal cell lines. Further, AMB caused increased accumulation of the HCC cells in subG1 phase, decreased the expression of Bcl-2, Bcl-xL, cyclin D1, caspase-3, survivin and increased the cleavage of PARP in a time-dependent manner. In silico molecular interaction studies between Bcl homologs and AMB showed that the biphenyl scaffold is predicted to form π–π interactions with Phe-101 and Tyr-105 and the adamantyl fragment is predicted to occupy another hydrophobic region in the kink region of the binding groove. In summary, we report on the synthesis and biological characterization of adamantyl-tethered biphenylic compounds that induce apoptosis in tumor cells most likely by targeting Bcl homologs.

Aged cells, damaged cells, auto-reactive cells and cells with irreparable DNA damage are eliminated from the body of all the multicellular organisms by a complex process of programmed cell death or apoptosis.[1] In multicellular organisms, a balance is maintained between the rate of cell division and cell death.[2] In case of oncogenesis, cells exhibit density independent proliferative potential and resistance to apoptotic signals.[3] Bcl-2 family proteins play a key role in modulation of apoptosis and more than 20 members of the Bcl-2 family have been discovered and broadly categorized into pro and antiapoptotic proteins.[4,5] BAD, BAK, BAX, PUMA, NOXA are proapoptotic and Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1 are predominant antiapoptotic proteins of the Bcl-2 protein family.[6–8] The equilibrium is maintained between pro and antiapoptotic proteins to ensure homeostasis.[9,10] Overexpression of antiapoptotic Bcl-2 family proteins has been demonstrated to contribute to prolonged cell survival, resistance to apoptosis, cancer development and progression, and decreased sensitivity to chemotherapeutics in cancer cells.[11,12] The pro-apoptotic BAD protein interact with the hydrophobic binding groove of Bcl homologs (Bcl-2 and Bcl-xL)[13] and disrupts the integrity of mitochondria to initiate the release of cytochrome C into the cytoplasm to induce apoptosis.[14,15] Therefore, targeting Bcl-2/Bcl-xL proteins using small molecules that can interact with their hydrophobic socket may serve as agents with therapeutic potential to induce apoptosis in cancer cells.

Several heterocycles targeting Bcl homologs have been extensively investigated and few of them have been promoted to clinics.[16,17] Navitoclax (ABT-263) is a well-characterized small molecule inhibitor of Bcl-2 that has been synthesized following a structure based drug design approach.[18] Further structure based

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design yielded the potent selective inhibitors of Bcl-2 called ABT-199 and ABT-737.[19,20] In addition, biphenyl based compounds have been studied extensively and were shown to exhibit very good antitumor activity against various human malignancies.[21,22] Honokiol and magnolol are the biphenyl containing natural compounds isolated from *Magnolia officinalis* with an excellent antineoplastic activity against several cancer types.[23,24] The synthetic derivatives of eugenol have been demonstrated to induce antiproliferative activity on neuroectodermal tumor cells.[25] However, the mechanism of action of these compounds is not clearly understood. In order to investigate the underlying mechanism of biphenyl derivatives in imparting anticancer activity and in continuation of our effort to synthesize various organic compounds[26–31], we synthesized a series of adamantyl tethered biphenyl derivatives. Adapalene is a topical drug in which adamantane substitution at *meta* position to the aryl system is seen. Therefore, we considered *meta* position as choice of substitution and synthesized biphenyl-adamantanes and evaluated for their antiproliferative activity against hepatocellular carcinoma cell lines. We also followed an in silico approach to explain the role of biphenyls in apoptosis induction of cancer cells.

The synthetic route for biphenyl-based small molecule libraries is outlined in Figure 1A. Here we attempted the synthesis of targeted compounds by a ligand free palladium on carbon based Suzuki reaction using diisopropylamine as a base for the coupling of adamantane based aryl chloride with a variety of aryl boronic acids.[32–34] Most of the reactions were completed in 4 h with excellent yields. On the other hand, ortho-substituted boronic acids showed prolonged reaction time with an exception of ortho methyl phenyl boronic acid which completed in 4 h. Interestingly, fused aromatic- and chloro substituted-boronic acids proved to be excellent substrates under the given experimental conditions. The characterization information and the structures of various biphenyl-adamantanes were provided as Supplementary Table 1.

AMB suppresses the viability of hepatocellular carcinoma (HCC) cells in a dose- and time-dependent manner: We investigated the potential effect of novel biphenyl derivatives on HepG2 cells using a MTT assay as described previously.[35–37] We found compounds **3f** (AMB), **3i** and **3m** to be potent cytotoxic agents against HepG2 cells and further evaluation revealed that the compounds **3i** and **3m** significantly induce cytotoxicity against normal hepatocytes (LO2) whereas AMB did not affect the viability of non-transformed cells. Therefore, 3′-(adamantan-1-yl)-4′-methoxy-[1,1′-biphenyl]-3-ol (**3f**, AMB) was found to be most effective cytotoxic agent among the newly synthesized compounds with the IC_{50} of 26.1 μM. Further, the effect of lead compound on the viability of a panel of three HCC cell lines (HepG2, Huh7 and Hep3B) at different dose (0, 10, 25, 37.5 and 50 μM) and time points (0, 24, 48 and 72 h) was also investigated. We found that AMB mitigated the proliferation of HepG2, Huh7 and Hep3B cell lines in a dose- and time-dependent manner (Fig. 1B). Navitoclax was used as positive control and observed the reduction in the percentage of cell viability in dose-dependent manner (Fig. 1B). All the synthesized compounds were screened for their cytotoxic activity on LO2 cells. However, except

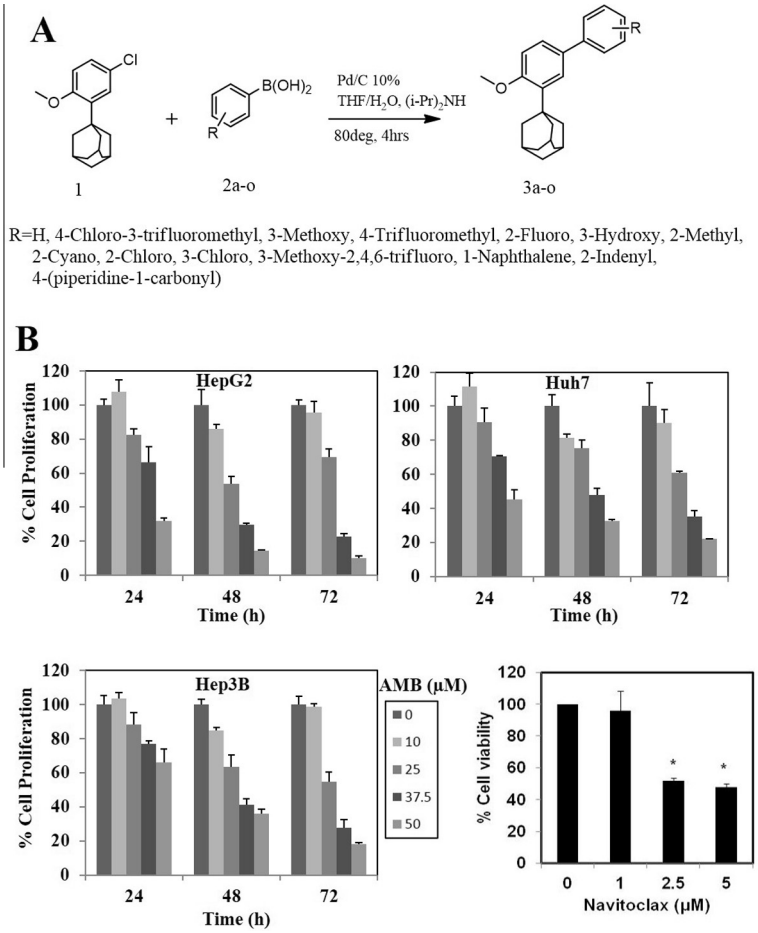

Figure 1. (A) Schematic representation for the synthesis of biphenyl based small molecules. (B) HCC cells (2.5×10^4/mL, HepG2, Huh7 and Hep3B) were plated in triplicate, treated with indicated concentrations of AMB, and then subjected to MTT assay after 24, 48 and 72 h to analyze proliferation of cells. AMB suppresses the viability of various HCC cell lines in a dose- and time-dependent manner. Navitoclax, a small molecule inhibitor of Bcl-2/Bcl-xL/Bcl-W also suppressed the viability of HepG2 cells in a dose-dependent manner $^*p < 0.05$.

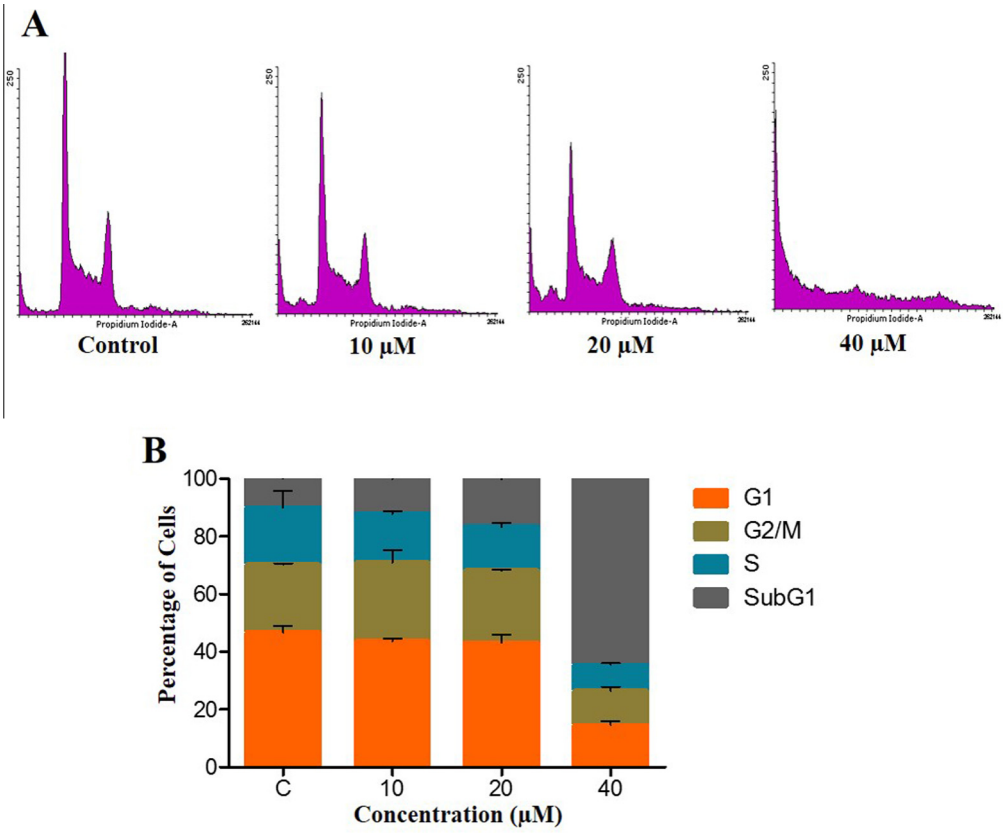

Figure 2. (A) HepG2 cells were treated with different concentrations of AMB and cell cycle distribution was analyzed by staining with propidium iodide. The flow cytometry analysis revealed that AMB accumulates HepG2 cells in the subG1 phase. (B) Bar diagram showing distribution of HepG2 cells in different phases of cell cycle after treatment with various concentrations of AMB.

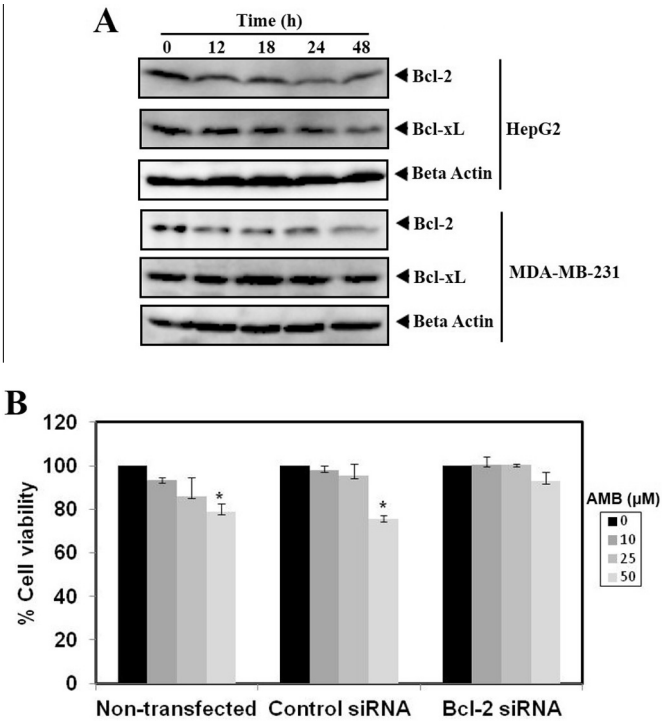

Figure 3. (A) Analysis of Bcl-2 and Bcl-xL expression in AMB treated HepG2 and MDA-MB-231 cells using western blot analysis. (B) Transfection of HepG2 cells with Bcl-2 siRNA results in the significant reversal of antiproliferative effect of AMB compared to cells transfected with control siRNA suggesting that AMB primarily exerts its cytotoxic effects by negative regulation of Bcl-2 protein $^{*}p < 0.05$.

compound 3i and 3 m, other biphenyl derivatives did not induce cytotoxicity on LO2 cells thereby indicating that synthesized compounds do not exert cytotoxic effect on normal cells (data not shown).

AMB causes increased accumulation of HepG2 cells in SubG1 phase: We next evaluated the effect of AMB on the cell cycle distribution of HepG2 cells using flow cytometry analysis as described earlier.[38,39] Initially, the cells were treated with different concentrations of AMB (0, 10, 20, and 40 μM) for 48 h, fixed with ethanol and stained with propidium iodide and analyzed for cell cycle distribution. We observed an increased accumulation of cells in the subG1 phase in the dose-dependent manner upon AMB treatment (Fig. 2A and B) and the results further confirm the proapoptotic effects of AMB.

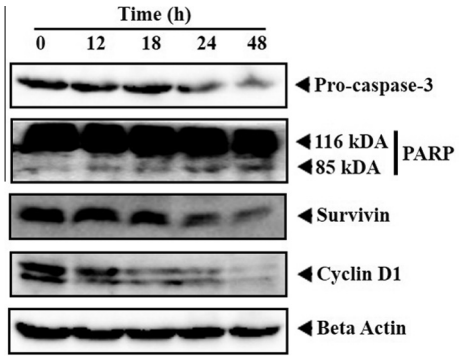

Figure 4. Profiling of expression of apoptotic markers (Caspase-3, PARP), antiapoptotic protein (Survivin), cell cycle regulator (Cyclin D1) in AMB treated HepG2 cells using Western blot analysis.

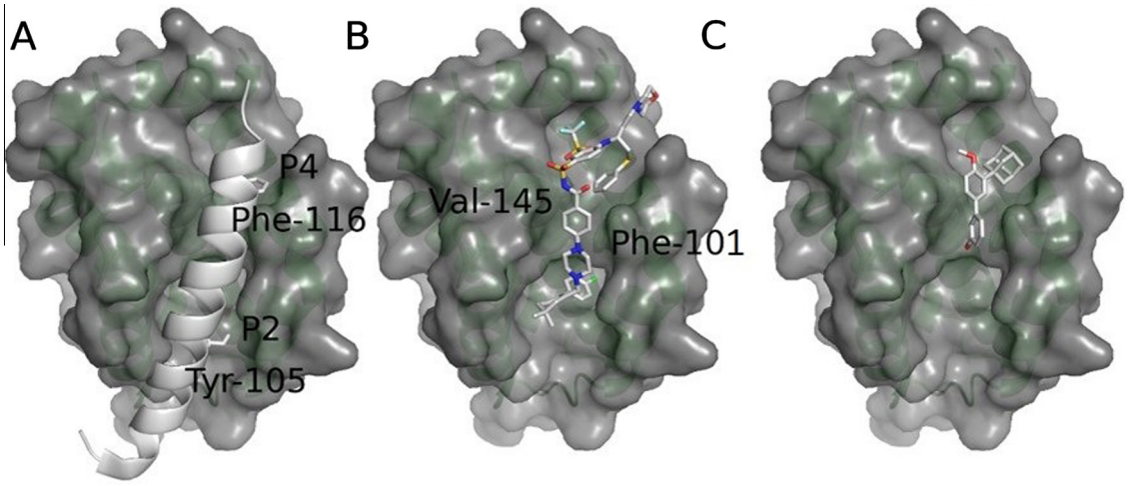

Figure 5. Predicted molecular interactions between Bcl2 and the biphenyl derivatives: Bcl proteins are shown in green cartoon representation with a semi-transparent surface. (A) A helix from BAD occupies a hydrophobic binding groove in the Bcl2 homolog Bcl-xL (PDB: 2BZW). (B) This binding site can be blocked by synthetic compounds as shown via the co-crystal structure of Bcl2 and navitoclax (PDB: 4LVT). C, AMB is predicted to occupy the same binding site, thereby using the biphenyl backbone as α-helix mimetic and positioning the adamantyl moiety in the P4 binding hot-spot.

AMB inhibits Bcl homologs in HepG2 and MDA-MB-231 cells: Navitoclax interacts with P2 and P4 hotspot of the Bcl-2/Bcl-xL proteins to serve as a potential inhibitor of Bcl homologs.[16] We further analyzed the expression Bcl-2 and Bcl-xL in AMB treated HepG2 cells at different time points (0, 12, 18, 24 and 48 h) using western blotting analysis as described earlier.[40,41] Our results showed that AMB clearly decreased the levels of Bcl-2 and Bcl-xL in the time dependent manner with maximum inhibition observed at 48 h (Fig. 3A). We also investigated the effect of compound AMB on the expression of anti-apoptotic protein Bcl-2 and Bcl-xL in breast cancer MDA-MB-231 cells. AMB substantially downregulated the expression of Bcl-2 in a time-dependent manner in MDA-MB-231 cells, whereas its potential effect on the Bcl-xL expression was less substantial as compared to that on Bcl-2. The levels of β-actin that was used as loading control remain unchanged upon AMB exposure (Fig. 3A). Next, we determined whether knockdown of Bcl-2 expression by siRNA could reverse the antiproliferative effect of AMB in HepG2 cells. Results shown in Figure 3B indicate that the observed antiproliferative effect of AMB on HepG2 cells was significantly reversed in cells transfected with Bcl-2 siRNA compared to cells transfected with control siRNA where there was a dose dependent decrease in cell proliferation (Fig. 3B). These results suggest that AMB primarily exerts its cytotoxic effects by negative regulation of Bcl-2 protein.

AMB induces apoptosis of HepG2 cells: Activation of caspases, cleavage of PARP and downregulation of antiapoptotic proteins is a hallmark event in the cells destined to undergo apoptosis.[42–44] During apoptosis, activated caspase-3 cleaves the PARP (116-kDa) into fragments of 85-kDa and 24-kDA to breakdown the DNA repair mechanism to drive the cell to apoptosis.[45,46] Therefore, we further evaluated the effect of AMB on the levels of pro-caspase-3, PARP, survivin and cyclin D1 in HepG2 cells. Figure 4 demonstrates the activation of pro-caspase-3 and concurrent decrease of full length PARP with an increase in cleaved fragment (85-kDa) in a time-dependent manner. We further noticed that treatment of AMB causes the downregulation of survivin (anti-apoptotic) and cyclin D1 (cell cycle regulator) proteins (Fig. 4) demonstrating the pro-apoptotic effects of the lead compound.

In silico docking predicted a common binding mode for all compounds of the adamantyl-tethered-biphenylic series: The whole series of compounds was docked to the co-crystal structure of Bcl-2 with the anticancer drug navitoclax (PDB: 4LVT)[16] using MOE.[47] Therefore, we prepared the protein structure using protonate3D from MOE[48] and ionized the ligands for physiological pH using MOE. Resulting poses were visualized using pymol[49] and molecular interactions were compared to the crystal structure of the protein–protein interface between Bcl-xL, a close homolog of Bcl2 and BAD (Fig. 5A).[13] The biphenyl scaffold of the compounds is consistently predicted to occupy the hydrophobic binding groove of Bcl-2 overlapping with the cognate ligand navitoclax (Fig. 5B). Therefore, complex formation with BAD and thus signaling would be inhibited. The biphenyl scaffold is predicted to form π–π interactions with Phe-101 and Tyr-105 in Bcl-2. Additionally, Van der Waals contacts to the aliphatic side-chain of Val-145 are formed. As the biphenyl scaffold occupied the space of the BAD helix, it can be considered an α-helix mimetic in agreement with literature reports.[50] The adamantyl fragment is predicted to occupy another hydrophobic region in the kink region of the binding groove (P4 hot-spot), thereby replacing the phenyl side-chain of Phe-116 of BAD. Different substitution patterns within the new series are predicted to interact with the P2 region of Bcl-2. Here, amide-linked fragments as shown for AMB maintain the position of polar groups comparable to the cognate ligand navitoclax (Fig. 5C). In conclusion, the overexpression of Bcl-2 family proteins is responsible for apoptotic resistance in various cancers. Therefore, it opens up an avenue for the design of small molecules with therapeutic potential by targeting Bcl-2 family members. Therefore, we synthesized and identified a novel biphenyl-adamantane based Bcl homolog inhibitors as biologically active compounds against human hepatoma cell lines.

Statistical analysis: Student *t*-test was used to analyze the data. $P < 0.05$ was considered statistical significant ($^{*}p < 0.05$; $^{**}p < 0.005$)

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.026.

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