Anti-Neoplastic Activity of 1,3-Diaza-2-Functionalized-Adamantan-6-One Compounds Against Melanoma Cells

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Abstract: Four series of 1,3-diaza-2-functionalized-adamantan-6-one derivatives, bearing at the 2 position SO, SO_2, POCl and PO_2H functional groups, were synthesized *via* a key quadruple Mannich reaction, followed by transformation of an aminal functionality into the final 2-thia- and 2-phospha compounds. The compounds were tested for cytotoxic activity against the mouse B16-F10 melanoma cell line. Malignant melanoma is notorious for its high resistance to chemotherapy, and new anti-melanoma drugs are urgently needed. The 2-thia compounds exhibited poor proliferation inhibition activity, but the 2-phospha derivatives showed significant activity, with IC_{50} values of 10-60 μM. The compounds induced cell death by G_2/M cell cycle arrest, which led to apoptosis, as determined by Annexin V-FITC/PI staining, mitochondrial membrane potential changes assessed by the JC-1 reagent, caspases 3 and 7 activation, and morphological changes.

Keywords: Diaza-adamantane, melanoma, anticancer drugs, cytotoxicity, apoptosis, cell cycle.

INTRODUCTION

Malignant melanoma is one of the most aggressive human cancers. Advanced melanoma may become metastatic, and has a very poor prognosis [1, 2]. Melanoma exhibits high resistance to chemotherapy and irradiation. Cancer cells may acquire drug resistance by improved DNA repair, drug clearance, and defects in the apoptotic pathways [3]. Unlike other resistant cancer types, melanoma is usually characterized by lack of p53 mutations [4]. The failure of anti-cancer therapy in melanoma is, at least in part, due to impaired apoptosis: down regulation of apoptotic proteins such as Apaf-1 [4-6], and over-expression of anti-apoptotic proteins (Bcl-2) [5,7,8] and survival proteins (survivin and NF-κB) [9-11]. Melanocytes secrete melanin and protect neighboring epidermal cells from DNA damage by UV light [12]. Therefore, it is not surprising that melanoma is also resistant to radiation therapy.

Although some small molecules were found to have activity against melanoma *in vitro* [11], for several years only one drug, Dacarbazine, was approved for the treatment of metastatic melanoma, with poor response (10-20% of the patients) and even lower rates of cure (about 5%) [13]. After a decade-long stagnation, three other drugs (Zelboraf [14, 15], Yervoy [16,17] and Peginterferon alfa-2b [18]) have been approved in 2011 for the treatment of late-stage melanoma. Their long-term effectiveness is still to be studied. Nevertheless, some limitations in their application are already evident. Zelboraf is only active against melanoma cells with the V600E and V600K mutations in B- Raf [19,20], which already excludes 40% of the patients. Furthermore, Zelboraf suffers poor response duration, and resistance mechanisms have already been observed [21]. Yervoy, a monoclonal antibody, is effective in only 25% of the patients. It may induce severe and even fatal immunological adverse effects resulting from T cell activation and proliferation [22, 23]. Thus, the development of anti-cancer agents for the treatment of melanoma, either alone or in combination with other drugs, is still of utmost importance.

1,3-Diaza-adamantane derivatives exhibit antibacterial [24], and psychotropic [25] activity. Other synthetic derivatives are sodium channel blockers [26] and κ-opiate and σ-receptor ligands [27]. Physiologically active natural alkaloids with the 1,3-diaza-adamantane structure are also known [28]. 1,3-Diaza-adamantan-6-one and 1,3-diaza-2-phospha-adamantan-6-one derivatives were previously evaluated against sarcoma, carcinoma and leukemia *in vivo*, though no mechanism was suggested for the observed activity [29]. Most of these compounds showed very low toxicity in mice. Some adamantane derivatives exhibited anti-melanoma activity, by inducing apoptosis *via* G_2/M cell cycle arrest [11]. These data indicate that adamantanes and their 1,3-diaza analogs are potential candidates for the development of anti-melanoma drugs. We previously synthesized a library of 3,7-diaza-bicyclo[3.3.1]nonan-9-ones (bispidinone) derivatives [30], which could serve as precursors for the synthesis of 1,3-diaza-adamantan-6-ones. Thus, in the present study we report on the cyclization of the parent 3,7-diaza-bicyclo[3.3.1]nonan-9-one compounds to produce four libraries of 2-thia- and 2-phospha-diaza-adamantanes, and on their *in vitro* anti-melanoma activity.

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MATERIALS AND METHODS

Chemistry

General

^{1}H NMR spectra were recorded at 200, 300 or 600 MHz, ^{13}C NMR spectra at 50.3, 75.5 or 150.9 MHz, ^{31}P NMR spectra at 81 MHz, and ^{19}F NMR spectra at 188.3 MHz, all in $CDCl_3$, unless otherwise specified. ^{1}H and ^{13}C chemical shifts are reported in ppm relative to TMS in $CDCl_3$ or relative to solvent resonance in other solvents; for other nuclei an external reference was used. Multiplicities designated as "d" and "t" refer to resonances with 2nd order character in AA'XX' systems. ^{13}C Signal multiplicity was determined by the DEPT technique. HMQC and HMBC methods were used to establish atom connectivities. HRMS was recorded in DCI^+ mode with methane as reagent gas, unless otherwise indicated. Chromatography refers to flash column chromatography, carried out on silica gel 60 (230-400 mesh ASTM, E. Merck). TLC was performed on E. Merck 0.2 mm percolated silica gel F-254 plates. Compounds were detected by UV light (254 nm) and/or by staining with vanillin spray reagent (1 g vanillin and 1 mL H_2SO_4 in 100 mL EtOH).

Ketones **1d-f**, 1,3-diaza-adamantan-6-ones **2a-f** and diAlloc-bispidinones **3a-f** were prepared as previously described [27].

General Procedure for the Synthesis of Diamines 4

Alloc-protected diamine **3** (1 mmol), $Pd(PPh_3)_4$ (0.02 mmol) and AcOH (2.4 mmol) were dissolved in CH_3Cl (20 mL) under argon atmosphere in a Schlenk tube equipped with a magnetic bar and fitted with a rubber septum,. The pale yellow color of the solution was then characteristic of the presence of the palladium (II) complex. Bu_3SnH (ca. 1.1 mmol) was then rapidly added to the stirred solution. A slightly exothermic reaction with vigorous gas evolution immediately ensued. At the end of the reaction (a few hours) the golden yellow color of the reaction mixture indicated the presence of palladium (0) species in the medium. The solution was diluted with $CHCl_3$ and extracted with aqueous $NaHCO_3$. The organic layer was acidified by H_2SO_4 (200 mL H_2O : 2 mL concentrated H_2SO_4), the aqueous layer was washed with $CHCl_3$, then made basic with NaOH 10% solution, and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$.

1,5-Dibutyl-3,7-diaza-bicyclo[3.3.1]nonan-9-one ***(4a)***. 66% yield. ^{1}H NMR δ 3.41 (d, J = 12.0 Hz, 4H, eq CCH_2N), 2.92 (d, J = 12.0 Hz, 4H, ax CCH_2N), 2.77 (bs, 2H, NH), 1.39-1.15 (m, 12H, Me(CH_2)$_3$), 0.90 (t, J = 7.1 Hz, 6H, Me); ^{13}C NMR δ 215.7, 59.8, 51.8, 31.7, 25.7, 23.7, 14.0; HRMS *m/z* for $C_{15}H_{29}N_2O$ (MH^+): calcd 253.2280, Found 253.2250.

1,5-Dipentyl-3,7-diaza-bicyclo[3.3.1]nonan-9-one ***(4b)***. 30% yield. ^{1}H NMR δ 3.43 (d, J = 12.0 Hz, 4H, eq CCH_2N), 3.01 (bs, 2H, NH), 2.93 (d, J = 12.0 Hz, 4H, ax CCH_2N), 1.33-1.19 (m, 16H, Me(CH_2)$_4$), 0.88 (t, J = 7.1 Hz, 6H, Me); ^{13}C NMR δ 215.3, 59.6, 51.7, 32.9, 31.9, 23.1, 22.6, 14.1; HRMS *m/z* for $C_{17}H_{33}N_2O$ (MH^+): calcd 281.2593, found 281.2597.

1,5-Diphenyl-3,7-diaza-bicyclo[3.3.1]nonan-9-one ***(4c)***. 81% yield. Mp: 259-260 °C ^{1}H NMR δ 7.38-7.26 (m, 10H, Ph), 3.88 (d, J = 12.0 Hz, 4H, CCH_2N), 3.71 (d, J = 12.0 Hz, 4H, CCH_2N); ^{13}C NMR δ 210.1, 138.3, 128.1, 127.5, 127.2, 61.3, 56.3; HRMS *m/z* for $C_{19}H_{20}N_2O$ (M^+): calcd 292.1576, found 292.1588.

1,5-Dibenzyl-3,7-diaza-bicyclo[3.3.1]nonan-9-one ***(4d)***. 27% yield. ^{1}H NMR δ 7.28-7.09 (m, 10H, Ph), 3.30 (d, J = 12.0 Hz, 4H, CCH_2N), 2.97 (d, J = 12.0 Hz, 4H, CCH_2N), 2.81 (s, 4H, CH_2Ph), 2.63 (bs, 2H, NH); ^{13}C NMR δ 214.3, 137.1, 130.6, 128.1, 126.4, 59.5, 52.2, 38.1; HRMS *m/z* for $C_{21}H_{25}N_2O$ (MH^+): calcd 321.1967, found 321.1968.

General Procedure for the Synthesis of Cyclic Sulfurous Diamides 5

To a chloroform solution (3 mL) of diamine **4** (0.13 mmol) and triethylamine (71 μL, 0.51 mmol) was added a solution of distilled $SOCl_2$ (14 μL, 0.19 mmol) in chloroform (1 mL) at 0 ^{o}C under N_2 atmosphere. After stirring at rt for 5 h, the mixture was extracted with water and chloroform (3x30 mL). The combined organic layer was washed with saturated NaCl solution. Drying over $MgSO_4$ and concentration in vacuum afforded the clean product.

5,7-Dibutyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2-oxide ***(5a)***. 92% yield. ^{1}H NMR δ 4.45 ("d", J = 14.4 Hz, 2H, CCH_2N), 3.90 ("d", J = 14.4 Hz, 2H, CCH_2N), 3.40 ("d", J = 14.4 Hz, 2H, CCH_2N), 2.83 ("d", J = 14.4 Hz, 2H, CCH_2N), 1.44-1.22 (m, 12H, Me(CH_2)$_3$), 0.93 (t, J = 6.9 Hz, 3H, Me), 0.92 (t, J = 7.1 Hz, 3H, Me); ^{13}C NMR δ 209.8, 60.1, 51.35, 47.0, 45.0, 31.6, 30.1, 25.4, 24.6, 23.6, 23.5, 14.0.

5,7-Dipentyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2-oxide ***(5b)***. 88% yield. ^{1}H NMR δ 4.45 ("d", J = 14.1 Hz, 2H, CCH_2N), 3.90 ("d", J = 14.4 Hz, 2H, CCH_2N), 3.40 ("d", J = 14.4 Hz, 2H, CCH_2N), 2.83 ("d", J = 14.4 Hz, 2H, CCH_2N), 1.47-1.22 (m, 16H, Me(CH_2)$_4$), 0.90 (t, J = 6.8 Hz, 3H, Me), 0.89 (t, J = 6.8 Hz, 3H, Me); ^{13}C NMR δ 209.8, 60.1, 51.36, 47.0, 45.1, 32.7, 32.6, 31.81, 30.4, 22.9, 22.5, 22.1, 14.1; HRMS *m/z* for $C_{17}H_{30}N_2O_2S$ (M^+): calcd 326.2028, found 326.1993.

5,7-Diphenyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2-oxide ***(5c)***. 100% yield. ^{1}H NMR δ 7.42-7.19 (m, 10H, Ph), 4.95 ("d", J = 14.4 Hz, 2H, CCH_2N) 4.42 ("d", J = 14.4 Hz, 2H, CCH_2N) 4.15 ("d", J = 14.4 Hz, 2H, CCH_2N) 3.59 ("d", J = 14.4Hz, 2H, CCH_2N); ^{13}C NMR δ 205.2, 136.5, 135.3, 128.7, 128.6, 128.1, 128.0, 127.0, 126.6, 60.7, 51.7, 50.5, 48.9; HRMS *m/z* for $C_{19}H_{18}N_2O_2S$ (M^+): calcd 338.1089, found 338.1085.

5,7-Dibenzyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2-oxide ***(5d)***. 99% yield. ^{1}H NMR δ 7.35-7.12 (m, 10H, Ph), 4.38 ("d", J = 14.1 Hz, 2H, CCH_2N), 3.81 ("d", J = 14.1 Hz, 2H, CCH_2N), 3.34 ("d", J = 14.4 Hz, 2H, CCH_2N) 2.88 (s, 2H, CH_2Ph), 2.80 ("d", J = 14.4 Hz, 2H, CCH_2N), 2.79 (s, 2H, CH_2Ph); ^{13}C NMR δ 208.8, 135.3, 135.1, 130.7, 130.5, 128.7, 128.6, 127.1, 127.0, 59.5, 51.2, 47.6, 45.4, 37.9, 36.3; HRMS *m/z* for $C_{21}H_{22}N_2O_2S$ (M^+): calcd 366.1402, found 366.1371.

5,7-Bis[(4-(trifluoromethyl)phenyl)methyl]-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2-oxide ***(5e)***. To **2e** (1.0 mmol) in pyridine : acetonitrile 1:1 (2 mL) was added a solu-

tion of distilled $SOCl_2$ (90 μL, 1.2 mmol) in acetonitrile (1 mL) at 0 °C. The solution was stirred over night at rt, followed by 1 h under reflux. The mixture was cooled, and poured into ice-water bath. The crude product was collected, washed with cold water, and purified by chromatography ($CHCl_3$ + 1% MeOH). Yield: 151.5 mg (30%). ^{1}H NMR δ 7.58 (d, *J* = 7.8 Hz, 2H, Ar, (H-3)), 7.55 (d, *J* = 7.8 Hz, 2H, Ar, (H-3)), 7.36 (d, *J* = 8.1 Hz, 2H, Ar (H-2)), 7.31 (d, *J* = 8.1 Hz, 2H, Ar (H-2)), 4.39 ("d", *J* = 14.1, Hz, 2H, CCH_2N), 3.82 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.35 ("d", *J* = 14.4 Hz, 2H, CCH_2N) 2.93 (s, 2H, CH_2Ar), 2.84 (s, 2H, CH_2Ar), 2.79 ("d", *J* = 14.4 Hz, 2H, CCH_2N); ^{13}C NMR δ 208.1, 139.6, 131.2, 131.0, 125.7 (q, $^3J_{CF}$ = 3.8 Hz), 125.5 (q, $^3J_{CF}$ = 3.7 Hz), 59.6, 51.2, 47.7, 45.6, 37.6, 36.2; HRMS *m/z* for $C_{23}H_{21}F_6N_2O_2S$ (MH^+): calcd 503.1228, found 503.1246.

General Procedure for the Synthesis of Cyclic Sulfamides 6

To diamine **4** (0.3 mmol) in $CHCl_3$ (5 mL) was added Et_3N (1.06 mmol). The solution was cooled to 0 °C, treated with SO_2Cl_2 (0.31 mmol), and stirred for 1 h at 0 °C, followed by 4 h at rt. After evaporation of the solvent, the mixture was diluted with $CHCl_3$ (40 mL) and washed with 0.5M H_3PO_4, saturated $NaHCO_3$ solution and brine, dried over $MgSO_4$ and evaporated.

*5,7-Dibutyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6a**).* 66% yield. ^{1}H NMR δ 3.21 (d, *J* = 10.5 Hz, 4H, eq CCH_2N), 3.00 (d, *J* = 10.2 Hz, 4H, ax CCH_2N), 1.56-1.16 (m, 12H, Me(CH_2)$_3$), 0.90 (t, *J* = 7.1 Hz, 6H, Me); ^{13}C NMR δ 207.6, 71.7, 59.2, 28.0, 26.7, 23.5, 13.9; HRMS *m/z* for $C_{15}H_{26}N_2O_3S$ (M^+): calcd 314.1664, found 314.1698.

*5,7-Dipentyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6b**).* 59% yield. ^{1}H NMR δ 3.22 (d, *J* = 11.1 Hz, 4H, eq CCH_2N), 3.01 (d, *J* = 11.1 Hz, 4H, ax CCH_2N), 1.69-1.26 (m, 16H, Me(CH_2)$_4$), 0.88 (t, *J* = 6.8 Hz, 6H, Me); ^{13}C NMR δ 71.9, 59.2, 32.7, 27.1, 25.6, 22.5, 14.1; HRMS *m/z* for $C_{17}H_{31}N_2O_3S$ (MH^+): calcd 343.2055, found 343.2078.

*5,7-Diphenyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6c**).* 85% yield. ^{1}H NMR δ 7.38-7.07 (m, 10H, Ph), 3.67 (s, 8H, CCH_2N); ^{13}C NMR δ 203.3, 133.9, 128.6, 128.4, 128.1, 71.6, 64.8; HRMS *m/z* for $C_{19}H_{18}N_2O_3S$ (M^+): calcd 354.1038, found 354.0998.

*5,7-Dibenzyl-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6d**).* 64% yield. ^{1}H NMR δ 7.31-7.09 (m, 10H, Ph), 3.15 (d, *J* = 10.8 Hz, 4H, CCH_2N), 2.87 (d, *J* = 10.8 Hz, 4H, CCH_2N), 3.01(s, 4H, CH_2Ph); ^{13}C NMR δ 206.6, 137.1, 130.1, 128.4, 126.6, 71.1, 59.3, 32.6; HRMS *m/z* for $C_{21}H_{22}N_2O_3S$ (M^+): calcd 382.1351, found 382.1315.

*5,7-Bis[(4-(trifluoromethyl)phenyl)methyl]-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6e**).* To 1,3-diaza-adamantan-6-one **2e** (1.0 mmol) in pyridine : acetonitrile 1:1 (2 mL) was added a solution of distilled SO_2Cl_2 (100 μL, 1.2 mmol) in acetonitrile (1 mL) at 0 °C. The solution was stirred overnight at rt, followed by 1 h under reflux. The mixture was cooled, and poured into ice-water bath. The crude was collected and washed with cold water. Purification by chromatography ($CHCl_3$ + 1% MeOH), afforded 45 mg (17% yield). ^{1}H NMR δ 7.56 (d, *J* = 8.1 Hz, 4H, Ar (H-3)), 7.32 (d, *J* = 8.1 Hz, 4H, Ar (H-2)), 4.25 (d, *J* = 13.7 Hz, 4H, CCH_2N), 3.22 (d, *J* = 13.7 Hz, 4H, CCH_2N), 2.91 (s, 4H, CH_2Ar); ^{13}C NMR δ 205.9, 139.1, 131.0, 125.8, 125.7, 60.9, 45.8, 35.4; HRMS *m/z* for $C_{23}H_{21}F_6N_2O_3S$ (MH^+): calcd 519.1177, found 519.1153.

*5,7-Bis[(3,5-bis(trifluoromethyl)phenyl)methyl]-1,3-diaza-2-thia-tricyclo[3.3.1.1^{3,7}]decan-6-one 2,2-dioxide (**6f**).* Was prepared as 6e in 17% yield. ^{1}H NMR δ **7.77 (s, 2H, Ar (H-4)), 7.72 (s, 4H, Ar (H-2))** 4.31 (d, *J* = 13.5 Hz, 4H, CCH_2N), 3.22 (d, *J* = 13.5 Hz, 4H, CCH_2N), 2.96 (s, 4H, CH_2Ar); ^{13}C NMR δ 205.4, 138.3, 130.9, 121.6 (septet, $^3J_{CF}$ = 3.7 Hz), 61.0, 46.0, 35.4; HRMS *m/z* for $C_{25}H_{19}F_{12}N_2O_3S$ (MH^+): calcd 655.0925, found 655.0927.

General Procedure for the Synthesis of Phosphorodiamidic Chlorides 7

To a chloroform solution (3 mL) of diamine **4** (0.13 mmol) and triethylamine (71 μL, 0.51 mmol) was added a solution of distilled $POCl_3$ (14 μL, 0.19 mmol) in chloroform (1 mL) at 0 °C under N_2 atmosphere. After stirring at rt for 5 h, the mixture was extracted with ether and chloroform and the combined organic layer was washed with brine. Drying over $MgSO_4$ and concentration in vacuum afforded the clean product.

*5,7-Dibutyl-2-chloro-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7a**).* 82% yield. ^{1}H NMR δ 4.41 ("d", *J* = 14.4, Hz, 2H, CCH_2N), 4.04 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.27 (d"d", *J* = 32.7, 14.4 Hz, 2H, CCH_2N), 3.22 (d"d", *J* = 26.7, 14.4 Hz, 2H, CCH_2N), 1.33 (m, 12H, Me(CH_2)$_3$), 0.93 (t, *J* = 6.6 Hz, 3H, Me), 0.91 (t, *J* = 6.8 Hz, 3H, Me); ^{13}C NMR δ 208.3, 61.3 (d, *J* = 2.6 Hz), 60.3 (d, *J* = 2.6 Hz), 49.2 (d, *J* = 6.5 Hz), 47.0 (d, *J* = 6.5 Hz), 29.9, 29.8, 25.5, 25.4, 23.6, 14.0, 13.9; HRMS *m/z* for $C_{15}H_{26}ClN_2O_2P$ (M^+): calcd 333.1499, found 333.1502.

*2-Chloro-5,7-dipentyl-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7b**).* 89% yield. ^{1}H NMR δ 4.41 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 4.03 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.27 (d"d", *J* = 33.0, 14.4 Hz, 2H, CCH_2N), 3.21 (d"d", *J* = 26.7, 14.4 Hz, 2H, CCH_2N), 1.44-1.21 (m, 16H, Me(CH_2)$_4$), 0.90 (t, *J* = 6.5 Hz, 3H, Me), 0.88 (t, *J* = 6.9 Hz, 3H, Me); ^{13}C NMR δ 208.3, 61.3 (d, *J* = 2.7 Hz), 60.3 (d, *J* = 2.7 Hz), 49.3, 47.1, 32.6, 32.5, 30.1, 23.0, 22.97, 22.9, 22.5, 14.1.

*2-Chloro-5,7-diphenyl-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7c**).* 100% yield. ^{1}H NMR δ 7.38-7.15 (m, 10H, Ph), 4.86 ("d", *J* = 14.4 Hz, 2H, CCH_2N) 4.48 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.95 (d"d", *J* = 27.0, 14.4 Hz, 4H, CCH_2N); ^{13}C NMR δ 203.8, 134.7, 134.6, 128.5, 128.4, 127.9, 127.8, 127.0, 126.7, 61.5 (d, *J* = 2.3 Hz), 60.5 (d, *J* = 2.3 Hz), 52.4 (d, *J* = 5.3 Hz), 50.4 (d, *J* = 5.3 Hz); ^{31}P-NMR δ 35.91 (quintet, *J* = 27 Hz); HRMS *m/z* for $C_{19}H_{19}ClN_2O_2P$ (MH^+): calcd 373.0873, found 373.0873.

*5,7-Dibenzyl-2-chloro-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7d**).* 88% yield. ^{1}H NMR δ 7.34-7.12 (m, 10H, Ph), 4.36 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.96 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.20 (d"d", *J* = 32.7, 14.4 Hz, 2H, CCH_2N), 3.16 (d"d", *J* = 26.7, 14.4 Hz, 2H, CCH_2N), 2.88 (s, 2H, CH_2Ph), 2.83 (s, 2H, CH_2Ph); ^{13}C NMR δ 207.4, 135.2, 130.6, 130.4, 128.7, 127.2, 127.1, 60.7

(d, *J* = 2.5 Hz), 60.2 (d, *J* = 2.5 Hz), 49.7 (d, *J* = 6.1 Hz), 47.4 (d, *J* = 6.7 Hz), 36.1, 35.8; ^{31}P NMR δ 35.86 (quintet, *J* = 29.4 Hz); HRMS *m/z* for $C_{21}H_{23}ClN_2O_2P$ (MH^+): calcd 401.1186, found 401.1233.

*5,7-Bis[(4-(trifluoromethyl)phenyl)methyl]-2-chloro-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7e**).* To 1,3-diaza-adamantan-6-one **2e** (1.0 mmol) in pyridine : acetonitrile 1:1 (2 mL) was added a solution of distilled $POCl_3$ (114 μL, 1.2 mmol) in acetonitrile (1 mL) at 0 ^oC. The solution was stirred over night at rt, followed by 1h under reflux. The mixture was cooled, and poured into ice-water bath. The crude was collected and washed with cold water. The product was purified by chromatography ($CHCl_3$ + 1% MeOH). 33% yield. ^{1}H NMR δ 7.57 ("t", *J* = 8.4 Hz, 4H, Ar), 7.34 ("t", *J* = 8.4 Hz, 4H, Ar), 4.38 ("d", *J* = 14.4, Hz, 2H, CCH_2N), 3.97 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.19 (d"d", *J* = 32.7, 14.7 Hz, 4H, CCH_2N) 2.93 (s, 2H, CH_2Ar), 2.88 (s, 2H, CH_2Ar); ^{13}C NMR δ 206.7, **139.5, 139.4, 131.1, 130.9, 129.5 (q, $^2J_{CF}$ = 42.2 Hz),** 125.6 **(q, $^3J_{CF}$ = 3.3 Hz),** 60.7 (d, *J* = 2.3 Hz), 60.1 (d, *J* = 2.1 Hz), 49.7 (d, *J* = 6.2 Hz), 47.5 (d, *J* = 6.5 Hz), 35.9, 35.7; ^{31}P NMR δ 35.44; HRMS *m/z* for $C_{23}H_{20}ClF_6N_2O_2P$ (M^+): calcd 536.0855, found 536.0848.

*5,7-Bis[(3,5-bis(trifluoromethyl)phenyl)methyl]-2-chloro-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decane-6-one 2-oxide (**7f**).* Was prepared as 7e in 31% yield. ^{1}H NMR δ 7.76 (m, 6H, Ar), 4.45 ("d", *J* = 14.4, Hz, 2H, CCH_2N), 4.01 ("d", *J* = 14.4 Hz, 2H, CCH_2N), 3.20 (d"d", *J* = 32.4, 14.7 Hz, 4H, CCH_2N) 2.98 (s, 2H, CH_2Ar), 2.95 (s, 2H, CH_2Ar); ^{13}C NMR δ **206.2, 137.8, 137.6, 132.1 (q, $^2J_{CF}$ = 33.2 Hz), 132.0 (q, $^2J_{CF}$ = 32.9 Hz),** 131.0, 130.8,125.0, 121.4, 60.9 (d. *J* = 1.8 Hz), 60.1 (d, *J* = 1.8 Hz), 49.8 (d, *J* = 5.9 Hz), 47.8 (d, *J* = 5.9 Hz), 35.9, 35.8; ^{31}P NMR δ 35.27; HRMS *m/z* for $C_{25}H_{18}ClF_{12}N_2O_2P$ (M^+) calcd 673.0681, found 673.0645.

*Sodium 5,7-dibutyl-6-oxo-1,3-diaza-2-phospha-tricyclo [3.3.1.1^{3,7}]decan-2-olate 2-oxide (**8a**).* To **7a** (0.3 mmol) in THF (2 mL) was added a solution of 2M NaOH (700 μL). The solution was stirred over night at reflux, and then concentrated under reduced pressure to give the pure product in 100% yield. ^{1}H NMR **(DMSO-d_6)** δ 3.89 (d, *J* = 13.0 Hz, 4H, eq CCH_2N), 2.69 (dd, *J* = 21.0, 12.4 Hz, 4H, ax CCH_2N), 1.21 (m, 12H, $Me(CH_2)_3$), 0.81 (t, *J* = 6.5 Hz, 6H, Me); ^{13}C NMR δ 211.9, 61.1, 47.4 (d, *J* = 5.0 Hz), 30.2, 25.2, 23.3, 13.9; ^{31}P NMR δ 3.39.

*Sodium 6-oxo-5,7-diphenyl-1,3-diaza-2-phospha-tricyclo [3.3.1.1^{3,7}]decan-2-olate 2-oxide (**8c**).* Was prepared as **8a** in 86% yield. ^{1}H NMR **(DMSO-d_6)** δ 7.38 (m, 10H, Ph), 4.42 (d, *J* = 12.8 Hz, 4H, CCH_2N), 3.95 (dd, *J* = 22.2, 12.8 Hz, 4H, CCH_2N); ^{13}C NMR δ 135.9, 128.6, 127.8, 126.9, 60.7, 51.8(d, *J* = 4.5 Hz); ^{31}P NMR δ 2.47; HRMS (MALDI-TOF) *m/z* for $C_{19}H_{18}N_2O_3P$ (M^-): calcd 353.1050, found 353.107.

*Sodium 5,7-dibenzyl-6-oxo-1,3-diaza-2-phospha-tricyclo [3.3.1.1^{3,7}]decan-2-olate 2-oxide (**8d**).* Was prepared as **8a** in 100% yield. ^{1}H NMR **(DMSO-d_6)** δ 7.19 (m, 10H, Ph), 3.84 (d, *J* = 12.8 Hz, 4H, eq CCH_2N), 2.71 (dd, *J* = 21.0, 12.4 Hz, 4H, ax CCH_2N), 2.65 (s, 4H, CH_2Ph); ^{13}C NMR δ 211.2, 137.4, 130.5, 127.9, 126.0, 60.6, 48.5 (d, *J* = 5.0 Hz), 36.0; ^{31}P NMR δ 2.48 (quintet, *J* = 21 Hz).

*Sodium 5,7-bis[(4-(trifluoromethyl)phenyl)methyl]-6-oxo-1,3-diaza-2-phospha-tricyclo[3.3.1.1^{3,7}]decan-2-olate 2-oxide (**8e**).* Was prepared as **8a** in 100% yield. ^{1}H NMR **(DMSO-d_6)** δ 7.62 (d, *J* = 8.1 Hz, 4H, Ar (H-3)), 7.41 (d, *J* = 7.8 Hz, 4H, Ar (H-2)), 3.84 (d, *J* = 12.3 Hz, 4H, CCH_2N), 2.75 (s, 4H, CH_2Ar), 2.74 (dd, *J* = 19.8, 13.2 Hz, 4 H, CCH_2N); ^{13}C NMR δ 142.7, 131.3, 124.7, 60.6, 48.7, 36.0; ^{31}P NMR δ 2.19; HRMS (MALDI-TOF) *m/z* for $C_{23}H_{20}F_6N_2O_3P$ (M^-): calcd 517.1110, found 517.1140.

Pharmacology

Cell Culture

B16-F10 melanoma cell line was obtained from the American Type Culture Collection (ATCC). The B16-F10 melanoma cells were cultured in RPMI 1640 medium (Biological Industries, Inc., Kibbutz Beit Haemek, Israel), supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin–nystatin, and 0.2% amphotericin. The cells were maintained at 37 ^oC and 5% CO_2 in a humid environment.

Cell Proliferation and Viability Assay

The effect of the various 1,3-diaza-adamantan-6-one compounds on cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. B16-F10 cells (5 x 10^3) were grown in RPMI-10% FCS on a 96-well microtiter plate and the 1,3-diaza-adamantan-6-one compounds (10-1000 mM in DMSO) were added to a final concentration of 10-1000 μM. DMSO (at 0.1% concentration) was also added to control wells, to account for the effect of the vehicle on the cells. After 24 h of incubation, the medium was replaced with 130 μl of fresh RPMI-1640 complete media and 20 μl of MTT reagent (5 mg/1 mL PBS) was added to each well. The cells were incubated at 37 ^oC for an additional 2 h. Cells were subsequently lysed by the addition of 100 μl DMF solution (50% final concentration of DMF and 20% SDS, pH 4.7) to each well, and incubated for 7 h. Absorption at 570 nm for each well was measured using an ELISA reader. IC_{50} values were calculated from cell viability data, and defined as the approximate concentration resulting in 50% inhibition of cell survival as compared to untreated cells.

Annexin V-FITC/PI Flow Cytometry Staining Technique

Apoptosis was determined with an Annexin V-FITC kit purchased from MBL Co. Ltd., according to the manufacturer's instructions. B16-F10 cells (6 x 10^5) were seeded in 100 mm culture dishes, and allowed to attach overnight. The cells were treated with 8e (50 μM, 60 μM, 75 μM) and 7e (25 μM, 30 μM) for 24 h, and then were collected and washed with ice-cold PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together. The washed cell pellet was resuspended in ice-cold binding buffer containing FITC-conjugated annexin V and PI. The sample was incubated for 15 min in the dark before analysis by flow cytometer.

JC-1 Mitochondrial Membrane Potential Detection Assay

The fluorescent cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethyl-benzimidazolylcarbocyanine iodide (JC-

1) was used for in situ detection of mitochondrial membrane transition events in live cells, to provide an early indication of the initiation of cellular apoptosis. JC-1 is internalized as a monomer in the cytosol (green fluorescence, emission wavelength 530 nm) and also accumulates as aggregates in mitochondria with negative inner membrane potential (red fluorescence, emission wavelength 590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. For this assay, cells were treated with **7e** (20 - 40 μM) and **8e** (50 - 100 μM) and maintained at 37 ^{o}C in a humidified 5% CO_2 atmosphere for 24 h. Cells were washed with PBS and centrifuged at 300 x g. The pellet was resuspended in 500 μl PBS, and 2 μl of 1 mg/mL JC-1 reagent was added for 20 min at 37 ^{o}C in the dark. Cells were washed with PBS and analyzed for apoptosis using a flow cytometer.

Caspase-3,7 Activity

Enzyme activity was measured using the carboxyfluorescein FLICA apoptosis detection kit caspase assay from Immunochemistry technologies, LLC with FAM-DEVD-FMK as a substrate, according to the manufacturer's protocols. B16-F10 cells (6.5 x 10^5) were plated in 100 mm culture dishes and grown for 24 h. **7e** (30 μM) and **8e** (75 μM) were added in RPMI-1640 complete media for 24 h. The medium was collected and the cells were washed with cold PBS. The cells were scraped and then washed twice by centrifugation at 500 x g for 5 min. The cell pellet was resuspended in 300 μl RPMI-1640 medium supplemented with 2 μM of 30 x FLICA solution and incubated for 1 h at 37 ^{o}C in the dark. Cells were washed with wash buffer and analyzed for apoptosis using a flow cytometer.

Flow Cytometry

B16-F10 cells (7 x 10^5) were seeded in 100 mm culture dishes, and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of **7e** (20 - 60 μM). Cells were incubated for 24 h at 37 ^{o}C, washed with PBS and centrifuged at 300 x g. Both the cells growing as a monolayer (harvested by scraping) and those floating in the medium were collected. The pellet was stained with 2μl PI solution for 15 min, and analyzed using a flow cytometer.

PI solution – To a mixture of 3 mL detergent 1% NP-40 and 15 mg RNase was added 10 mL water. The solution was stirred at 100 ^{o}C for 15 min. To the solution was added 2.5 mg PI (propidium iodide) and 37 mL water. The solution was stored at 4 ^{o}C in the dark.

RESULTS

Chemistry

1,3-diaza-adamantanones can be obtained via a quadruple Mannich reaction between a ketone, an aldehyde and ammonia [31]. A practical and efficient procedure involves the interaction of a ketone with hexamethylenetetramine [32]. Thus, a small library of six 5,7-dialkyl-1,3-diaza-adamantan-6-ones **2a**-**f** was prepared in this way in moderate to excellent (45-95%) yields (Scheme **1**). These compounds were transformed into the corresponding 1,5-dialkyl-3,7-diAlloc-3,7-diaza-bicyclo[3.3.1]nonan-9-one derivatives **3a**-**f**, typically in over 90% yield, and then were deprotected [33] to yield **4a**-**f**. These diamine compounds were then cyclized to a library of diaza-adamantane derivatives bearing six 5,7-dialkyl and diaryl substituents and three functional groups at the 2 position, SO (**5a**-**f**) [34], SO_2 (**6a**-**f**) [35,36], and POCl (**7a**-**f**) [37]. The latter was hydrolyzed to yield the fourth functional group PO_2^- (**8a**-**f**) (Scheme **1**). Alternatively, the aminal functional group of the diaza-adamantane derivatives **2** could be directly functionalized, to yield compounds **5**-**7** (Scheme **1**). This synthetic route is two steps shorter, but the functionalization step provided the final products in lower yields (17-33% yield). It was applied to the fluorinated aryl derivatives **5e**, **6e**,**f** and **7e**,**f**.

Some of the ketone starting materials **1** were not commercially available, and therefore had to be synthesized (Scheme **2**). They were prepared either by reduction of an unsaturated analog (Scheme **2a**) or by a one-pot oxidation-condensation-reduction reaction sequence between acetone and an alcohol, catalyzed by an iridium complex (Scheme **2b**) [38].

Pharmacology

Cytotoxic Activity

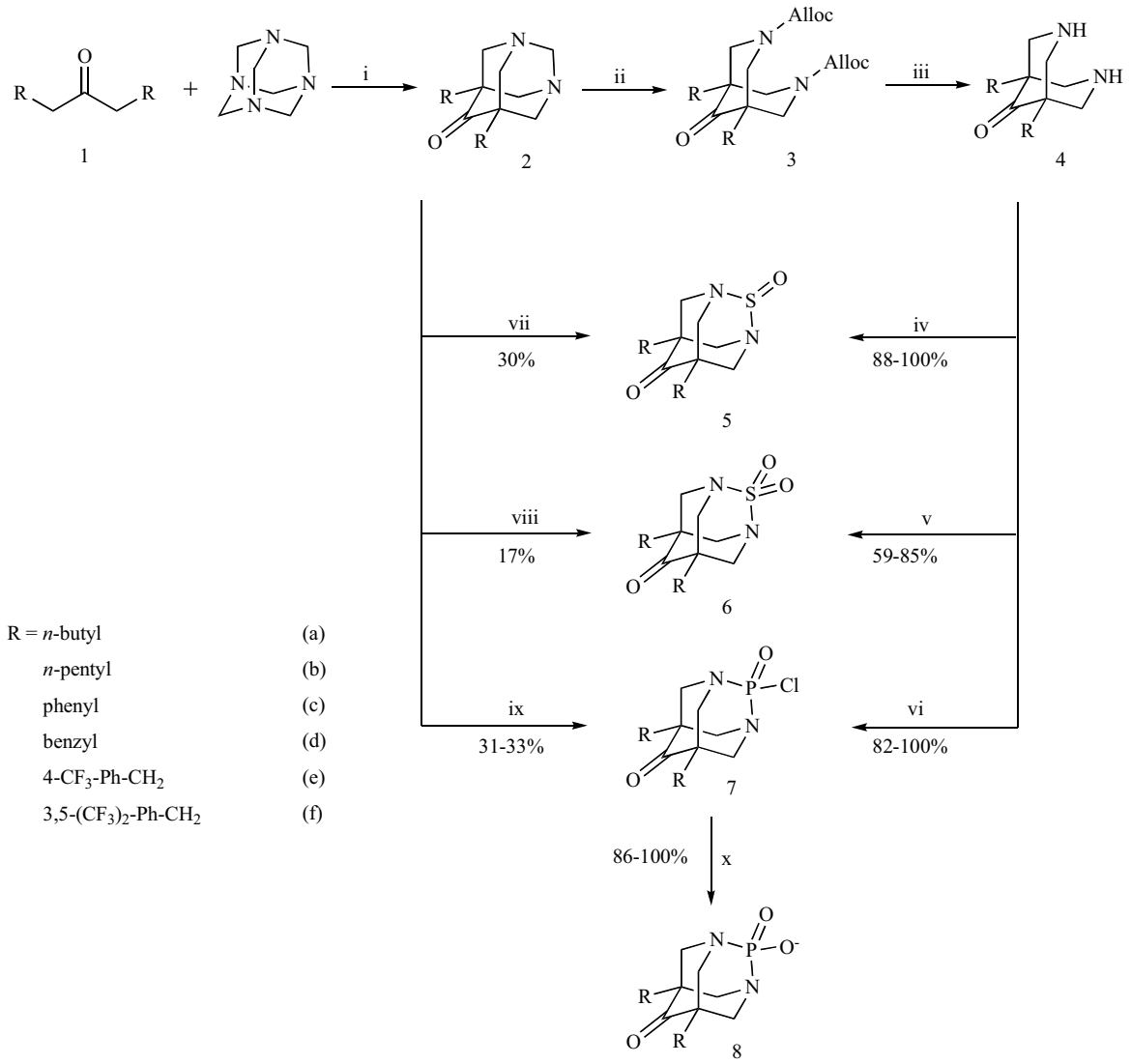
Representatives of the four families of functionalized 1,3-diaza-adamantan-6-ones **5**-**8** were tested *in vitro* for proliferation inhibition of mouse B16-F10 melanoma cells. The test is based on the MTT assay for mitochondrial dehydrogenases activity of live cells. The 1,3-diaza-2-thia-adamantan-6-one derivatives **5** and **6** showed poor anti-melanoma activity (IC_{50} > 200 μM). On the other hand, both acid chloride **7** and acid **8** derivatives of 1,3-diaza-2-phospha-adamantan-6-one exhibited significant anti-melanoma activity, with IC_{50} values in the 10-60 μM range (Fig. **1**).

Effect of 1,3-diaza-adamantan-6-ones on Cell Cycle

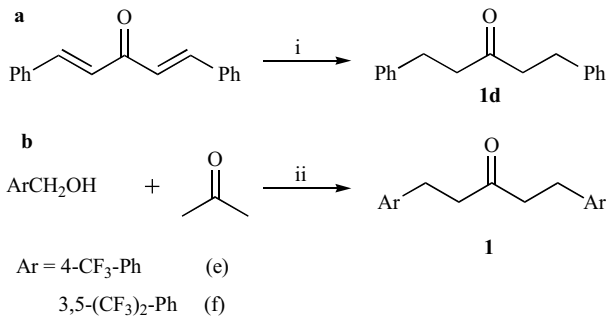
To gain further insight into the mechanism by which cell reduction is achieved, we applied fluorescence-activated cell sorting (FACS) analysis to study the effect of one of the compounds on cell cycle distribution (Fig. **2**). A 24-h incubation of B16-F10 cells with 20 μM **7e** induced enrichment of cells in the G_2/M phase. Higher doses of **7e** (up to 60 μM) induced a profound sub-G1 peak at the expense of cells at all other phases.

Induction of Apoptosis

We selected compounds **7e** and **8e** (5,7-dibenzyl-2-chloro-1,3-diaza-2-phospha-adamantan-6-one 2-oxide and 5,7-dibenzyl-2-hydroxy-1,3-diaza-2-phospha-adamantan-6-one 2-oxide, respectively), representing the two families of the acid chloride and acid derivatives of 1,3-diaza-2-phospha-adamantane, to further investigate the effect of the 1,3-diaza-adamantane compounds on the melanoma cells. These were among the best proliferation inhibitors (IC_{50} values of 25 μM and 60 μM, respectively), and they bear the same 5,7-dibenzyl substitution pattern so the effect of the 2-functional group could be directly evaluated.



Scheme (1). Synthetic scheme for the library of 1,3-diaza-2-functionalized-adamantan-6-one derivatives **5-8**. (i) AcOH (cat), EtOH, reflux, 15 h. (ii) allyl chloroformate, $CHCl_3$, rt, 5 days. (iii) AcOH, $Pd(PPh_3)_4$, Bu_3SnH, $CHCl_3$, rt, 4 h. (iv) $SOCl_2$, Et_3N, $CHCl_3$, rt, 5 h. (v) SO_2Cl_2, Et_3N, $CHCl_3$, rt, 4 h. (vi) $POCl_3$, Et_3N, $CHCl_3$, rt, 5 h. (vii) $SOCl_2$, pyridine, CH_3CN, rt, 15 h. (viii) SO_2Cl_2, pyridine, CH_3CN, rt, 15 h. (ix) $POCl_3$, pyridine, CH_3CN, rt, 15 h. (x) NaOH (2 M, aq), THF, reflux, 15 h.



Scheme (2). Synthesis of ketone **1d-f** starting materials. (i) H_2 5 atm, Pd-C 10%, AcOH, EtOAc, 7 h. (ii) $[IrCl(cod)]_2$, PPh_3, KOH, 100 ºC, 5-7 h.

We carried out three sets of experiments to assess apoptotic cell death: quantification of apoptotic cells by the annexin V-FITC/PI assay, identification of mitochondrial membrane potential changes by the fluorescent cationic dye 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolocarbocyanin iodide (JC-1 reagent) [39], and fluorescent-labeled inhibitor of caspases (FLICA)-based quantification of induced caspase 3 and 7 activity. Fig. (**3**) shows the effect of 24 h treatment of B16-F10 melanoma cells with compounds **7e** (at 25 and 30 μM) and **8e** (at 50-75 μM). Under the specific conditions employed, **7e** at 30 μM induced 28% apoptosis, whereas most of the cells (82%) underwent either early- or late apoptosis when incubated with 75 μM of **8e**. Identical results were obtained from the JC-1 experiment, quantifying the percentage of cells with low membrane potential (apoptotic cells) (Fig. **4**).

Apoptotic cell death may be initiated through the death receptor (extrinsic) pathway, involving caspase 8 activation [40], or through the mitochondrial (intrinsic) pathway and caspase 9 activation [41]. Both pathways lead to activation of caspases 3, 6, and 7 [42]. Thus, the effect of 1,3-diaza-2-phospha-adamantan-6-ones **7e** and **8e** on B16-F10 melanoma

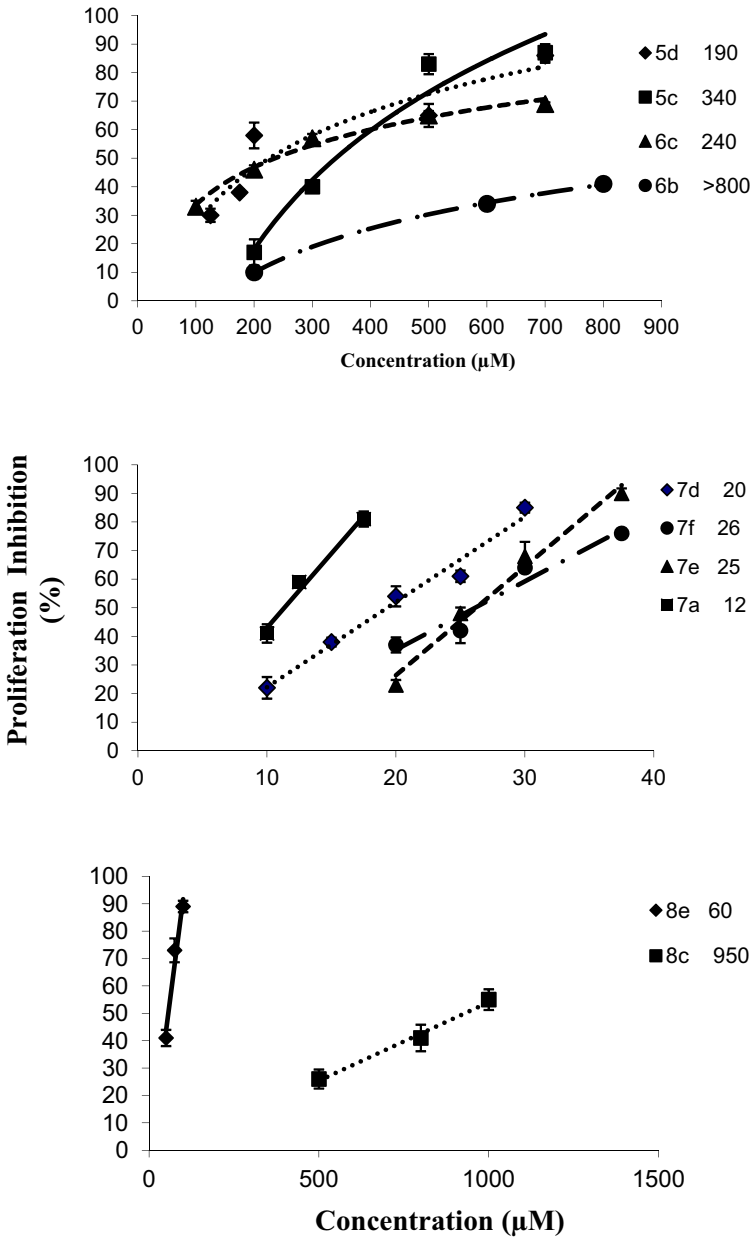


Fig. (1). Inhibition of melanoma B16-F10 cells proliferation by 1,3-aza-2-functionalized-adamantan-6-ones **5**-**8**. B16-F10 cells (5 x 10^3) were grown in a 96-well microtiter plate for 24 h, and then incubated with the 1,3-diaza-adamantane compounds (10-1000 mM in DMSO) at final concentration of 10-1000 μM. After 24 h, cell proliferation was measured by a modified MTT assay. Data is presented as % proliferation inhibition in comparison with control cells treated with vehicle only (0.1% DMSO). IC_{50} values are listed in the graphs. The experimental points are presented as an average ± S.D. of three independent experiments. The lines are for clarity only.

cells was also evaluated by measuring the induction of caspases 3 and 7 activity. Thus, 24 h incubation of the melanoma cells with 30 μM of **7e** lead to increased caspases activity in 56% of the cells. Likewise, 24 h incubation with **8e** (75 μM) induced caspase activity in 67% of the cells (Fig. **5**).

DISCUSSION

Chemistry

A one-pot quadruple Mannich reaction afforded 5,7-dialkyl/aryl-1,3-diaza-adamantan-6-one compounds that were transferred either directly or *via* a short sequence of aminal opening and amine protection / deprotection / functionalization to the target compounds. Thus, a library of 1,3-diaza-adamantan-6-one compounds, substituted at the 5 and 7 positions with alkyl or aryl groups and functionalized at the 2 position was prepared. The 2-functional groups include sulfurous amide (N_2SO, **5**), sulfamide (N_2SO_2, **6**), phosphorodiamidic chloride (N_2POCl, **7**), and phosphorodiamidic acid (N_2PO_2H, **8**) (Fig. **1**). The simplicity and the high yield of the reactions make the presented synthetic methodology suitable for the preparation of combinatorial libraries of 1,3-diaza-adamantan-6-ones, with varying functional groups and substituents.

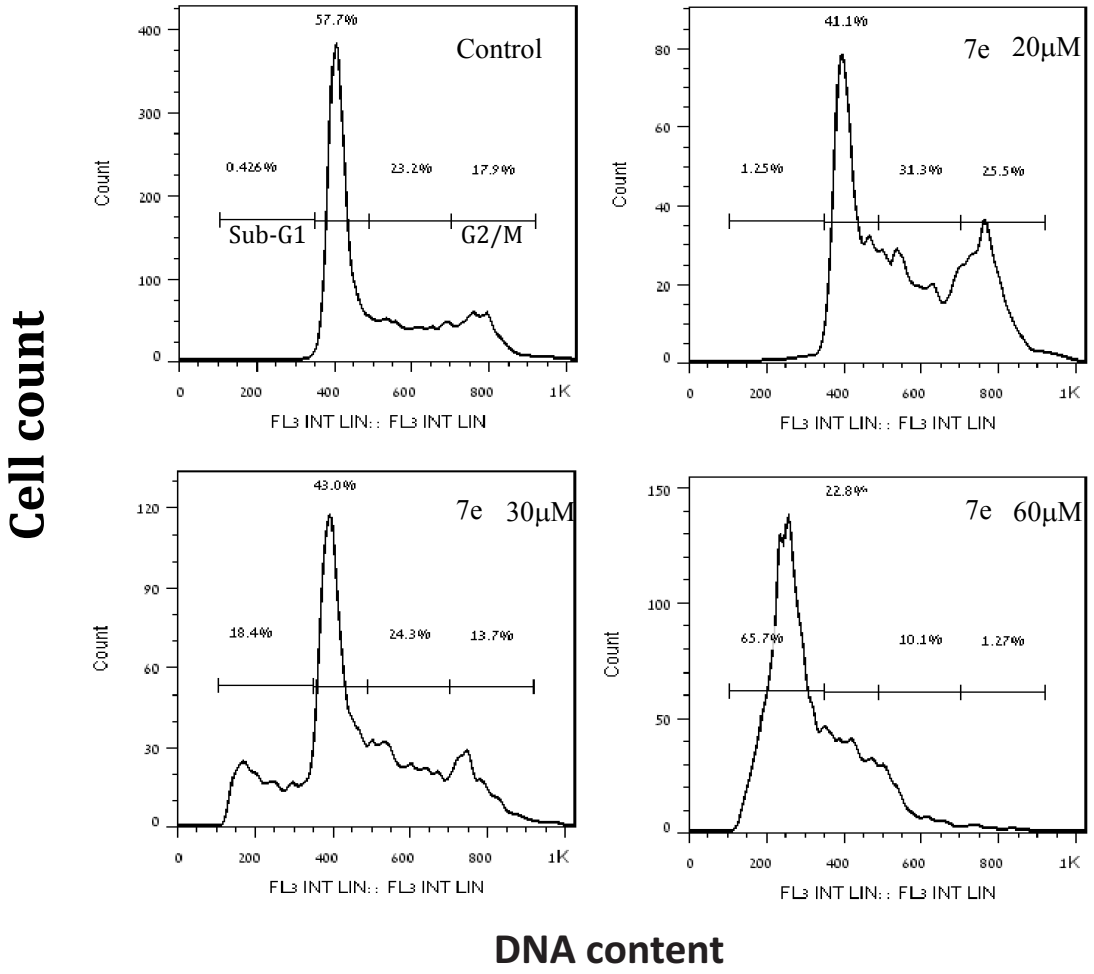


Fig. (2). The effect of 1,3-diaza-2-phospha-adamantan-6-one **7e** on cell-cycle distribution. B16-F10 cells were incubated with **7e** (20, 30 and 60 μM) for 24 h, and analyzed by DNA flow cytometry. The histograms show number of cells per channel (vertical axis) vs. DNA content (horizontal axis). The values indicate the percentage of cells in the indicated phases of the cell cycle. The data shown are representative of three independent experiments with similar results.

Pharmacology

Malignant melanoma is a highly aggressive tumor that frequently resists chemotherapy. Poor response, population selectivity, limited response duration and other limitations of the current drugs emphasize the urgent need to develop new effective anti-melanoma drugs. Thus, in light of the activity of similar compounds in other types of cancer [29], we tested the *in vitro* anti-melanoma activity of the library of compounds we synthesized on mouse B16-F10 melanoma cell line. The library can be divided into two groups: the group of 1,3-diaza-2-thia-adamantan-6-one derivatives **5** and **6** exhibited very poor anti-melanoma activity (IC_{50} > 200 μM). On the other hand, the two families of 2-phospha analogs **7** (phosphoryl chloride) and **8** (phosphate) exhibited much better proliferation inhibition activity, with IC_{50} values as low as 10 μM. Two compounds, **7e** and **8e**, were selected for mechanistic studies, since they vary in their functional group at the 2 position, but share the same 5,7 substitution. The inhibitory activity of **7e** is due to G_2/M arrest of the cell cycle. The cell cycle arrest induces apoptosis, as demonstrated by a set of various experiments (for both **7e** and **8e**), including specific apoptotic staining, mitochondrial membrane potential assessment and quantification of caspases activation. Apoptosis was also demonstrated by changes in the cell morphology (data not shown).

It is too early to speculate about the molecular mechanisms underlying the cytotoxic activity of the 1,3-diaza-adamantan-6-one derivatives. On one hand, various adamantane derivatives act as sodium [26] and potassium [43] channel blockers and as receptor ligands [27]. On the other hand, the diaza-adamantanone derivatives investigated in this study, especially the phosphorodiamidic chloride derivatives **7**, are hydrophobic molecules bearing an electrophilic functionality. As such they are reminiscent of some sesquiterpene lactones that exhibit anti-melanoma activity by modulating the concentration of cell cycle regulatory proteins and reducing the expression of cell survival proteins [44]. Further studies will shed light on the operative mechanisms. Future studies of the interaction of the new compounds with their biological target at a molecular level may also explain the large difference in the activity of the four functional groups presented in this study. At this stage it is not clear whether this difference arises from different uptake by the cells or by

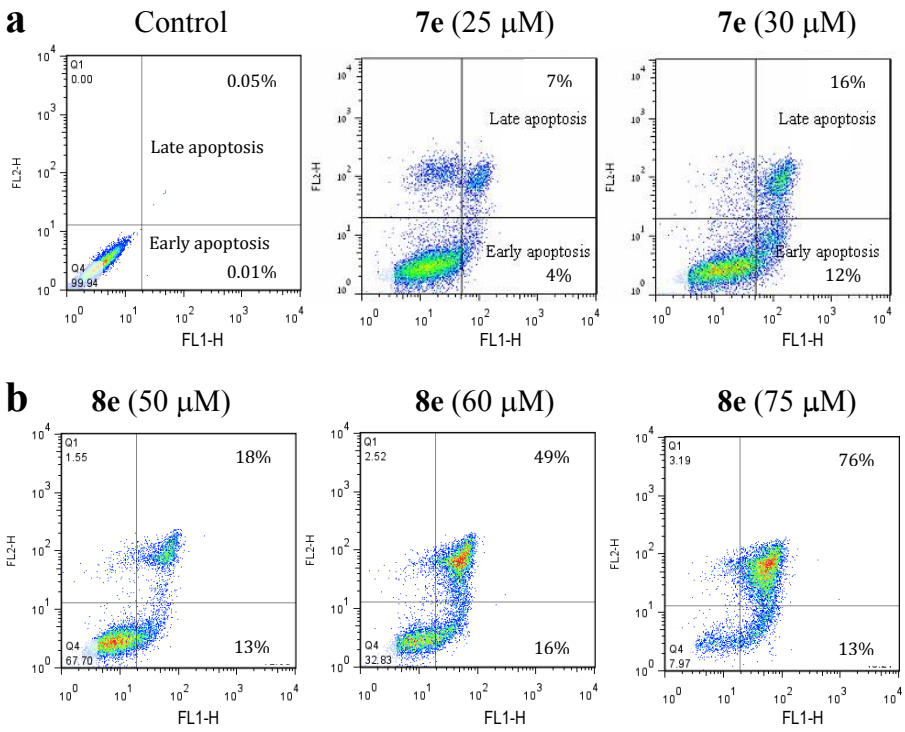


Fig. (3). Annexin V-FITC binding and propidium iodide uptake induced by 1,3-diaza-2-phospha-adamantan-6-ones **7e** and **8e**. B16-F10 cells were treated with (a) compound **7e** (25 and 30 μM) and (b) compound **8e** (50, 60 and 75 μM) for 24 h, stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. The control cells were incubated with the vehicle (0.1% DMSO). The horizontal (FL1-H) and vertical (FL2-H) axes represent labeling with Annexin V-FITC and PI, respectively. The data shown are representative of three independent experiments with similar results.

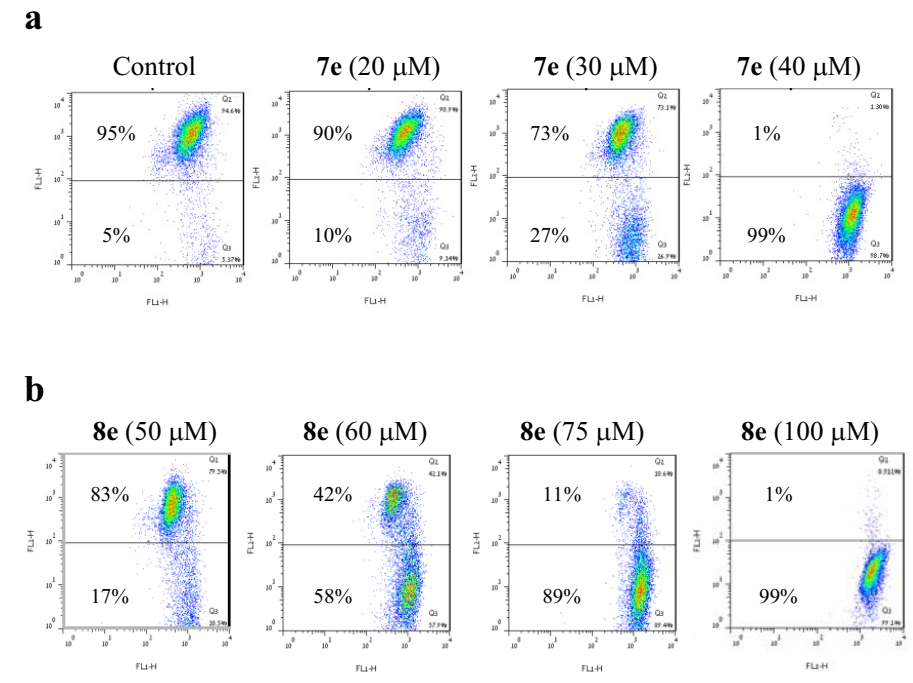


Fig. (4). The effect of 1,3-diaza-2-phospha-adamantan-6-ones **7e** and **8e** on mitochondrial membrane potential. B16-F10 cells were treated with (a) compound **7e** (20-40 μM) and (b) compound **8e** (50-100 μM) for 24 h to follow the extent of apoptosis by determination of mitochondrial membrane potential using JC-1 reagent. The control cells were incubated with the vehicle (0.1% DMSO). Cells were analyzed on a FACScan cytometer. Dot plots of red (FL2-H) vs. green fluorescence (FL1-H) show live cells with intact mitochondrial membrane potential and dead cells with lost mitochondrial potential, respectively. The data shown are representative of three independent experiments with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

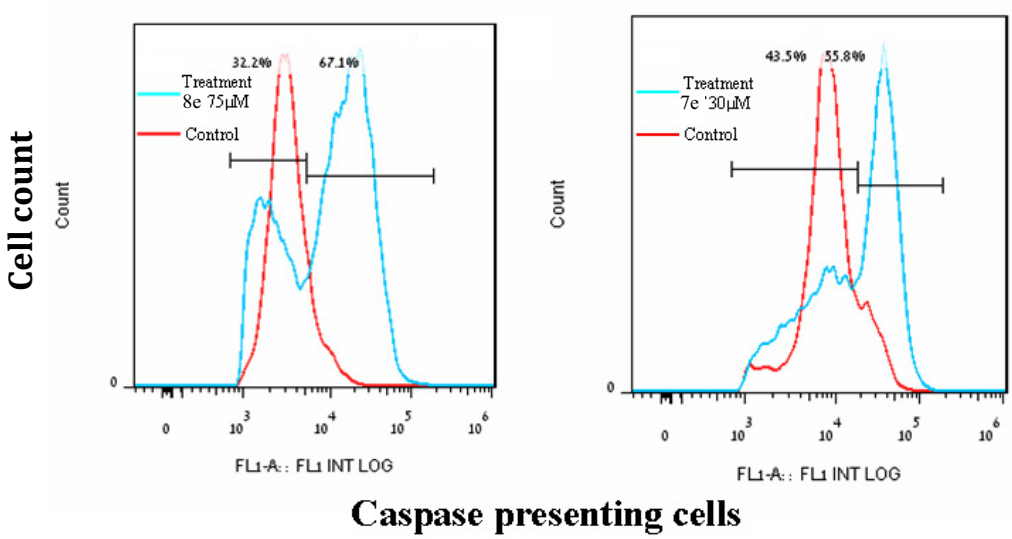


Fig. (5). The effect of 1,3-diaza-2-phospha-adamantan-6-ones **7e** and **8e** on caspases 3 and 7 activities. B16-F10 Cells were incubated with 30 µM **7e** (right) and 75 µM **8e** (left) for 24 h and their caspase 3 and 7 activities were measured. The control cells were incubated with the vehicle (0.1% DMSO). Enzymatic activity was determined with FAM-DEVD-FMK FLICA reagent and analyzed by flow cytometry. The frequency of events (Y axis) vs. fluorescence intensity (X axis) graph shows 2 peaks: caspase negative cells occur to the left (unlabeled cells) whereas caspase positive cells lay within the region in the right. The data shown are representative of three independent experiments with similar results.

different chemical reactivity towards the actual biological target.

The ability of the 1,3-diaza-2-phospha-adamantan-6-one compounds to induce apoptosis in melanoma cells is significant, considering the fact that such malignant cells are notorious for their resistance to apoptosis due to activation of cell survival strategies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

FACS = Fluorescence-activated cell sorting

FITC = Fluorescein isothiocyanate

FLICA = Fluorescent-labeled inhibitor of caspases

JC-1 = 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethyl-benzimidazolylcarbocyanine iodide

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

PI = Propidium iodide.

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