Tumor Necrosis Factor-α Production-Enhancing Properties of Novel Adamantylalkylthio Derivatives of Some Heterocyclic Compounds

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Certain adamantylated heterocycles were previously shown to enhance the secretion of tumor necrosis factor alpha (TNF-α) by murine melanoma cells that have been transduced with the gene for human TNF-α and constitutively expressed this cytokine. The stimulatory potency of those compounds depended, among other factors, on the structure of the linker between the adamantyl residue and the heterocyclic core. In the present study, a series of (1-adamantyl)alkylsulfanyl derivatives of heterocyclic compounds was prepared by alkylation of the corresponding thioheterocyles. Of the novel adamantylalkylthio compounds tested in the aforementioned cell line, 2-(2-adamantan-1-yl-ethylsulfanyl)-4-methyl-pyrimidine was found to be the most active.

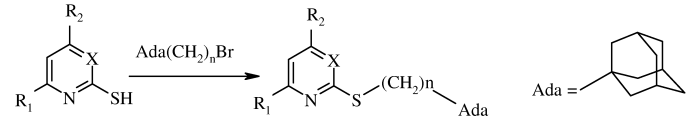
Key words: TNF-α, Adamantylated Pyridines, Adamantylated Pyrimidines, Cytotoxicity

Introduction

Tumor necrosis factor-alpha (TNF-α) is a cytokine produced mainly by activated monocytes/macrophages, and is an attractive target for the development of biological response modifiers (BRMs) [1, 2]. *In vitro* as well as *in vivo* studies on clinically relevant properties of TNF-α have shown both unfavorable and beneficial effects. The latter include direct antitumor activity because TNF-α exhibits selective cytotoxicity against various tumor cells. On the other hand, the overproduction of TNF-α has been related to some pathological conditions, *e.g.* of septic shock, multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis [3 – 5]. The medical aspects of TNF-α actions were the topic of some reviews most recently [6 – 8].

Lately, *N*-(1-adamantyl)phthalimide was reported to enhance TNF-α production greatly in 12-*O*-tetradecanoylphorbol-13-acetate-stimulated human leukemia HL-60 cells [9]. We have previously reported that numerous adamantane heterocyclic derivatives, primarily adamantylamino-pyrimidines, -pyridines and adamantylated phthalimides, enhance TNF-α production in human TNF-α gene-transduced murine melanoma B78 cells (B78/TNF) [10-12]. Similar activity was observed in this test system also for a variety of adamantylthio-heterocycles [13, 14]. However, attaching a rigid *N*-adamantylcarboxamide group to the respective heterocyclic core dramatically reduced the potency of the analog for the stimulation of TNF-α secretion by B78/TNF cells [14]. Our studies allowed us to put forth the hypothesis that both the structure of the heterocycle and the existence of a flexible linker (*e.g.* an amino group or sulfur atom) between the heterocyclic core and the adamantyl residue are of great importance for the biological activity of the adamantylated derivatives [14]. Of the variety of adamantylated heterocyclic compounds that have been studied since that time 2-(1-adamantylamino)-6-methylpyridine (**AdAmP**, Fig. 1) and 2-(1-adamantylthio)-6-methylpyridine (**AdtP, 2bs)** appeared the most potent [10, 14].

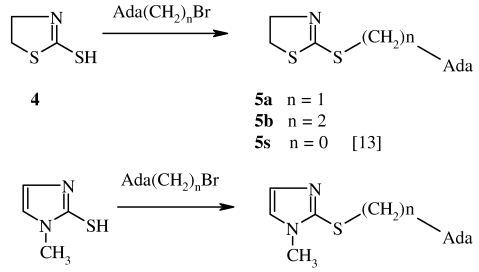
The present study was aimed at exploring the relationship between the flexibility of the link connecting the heterocycle and the adamantyl residue, and the TNF-α production-enhancing properties. We have



1a X = C, R_1, R_2 = H
1b X = C, R_1 = CH_3, R_2 = H
1c X = N, R_1, R_2 = H
1d X = N, R_1 = CH_3, R_2 = H
1e X = N, R_1, R_2 = CH_3

2a X = C, R_1, R_2 = H, n = 1
2b X = C, R_1 = CH_3, R_2 = H, n = 1
2c X = N, R_1, R_2 = H
2d X = N, R_1 = CH_3, R_2 = H
2e X = N, R_1, R_2 = CH_3
3a X = C, R_1, R_2 = H, n = 2
3b X = C, R_1 = CH_3, R_2 = H, n = 2
3c X = N, R_1, R_2 = H, n = 2
3d X = N, R_1 = CH_3, R_2 = H, n = 2
3e X = N, R_1, R_2 = CH_3, n = 2

2as X = C, R_1, R_2 = H, n = 0 [14]
2bs X = C, R_1 = CH_3, R_2 = H, n = 0 [14]
2cs X = N, R_1, R_2 = H, n = 0 [14]
2ds X = N, R_1 = CH_3, R_2 = H, n = 0 [14]
2es X = N, R_1, R_2 = CH_3, n = 0 [14]



4

5a n = 1
5b n = 2
5s n = 0 [13]

6

7a n = 1
7b n = 2
7s n = 0 [13]

Scheme 1.

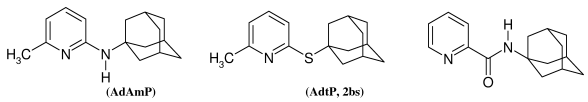


Fig. 1. The chemical structures of the two most active adamantane heterocyclic derivatives (**AdAmP**, and **AdtP**), and of the biologically inactive 2-(1-adamantylcarboxamido) pyridine.

focused on two such spacers: the methylenethio and ethylenethio groups. The heterocycle part was selected based on the properties of numerous previously synthesized compounds that showed high biological activity. *C*-methylated 2-thiopyrimidines, 2-thiopyridines, 1-methyl-2-thioimidazole and dihydrothiazole were used to further modify the structure of the compounds of interest.

Results and Discussion

The introduction of adamantylalkyl groups into the selected heterocycles was realized by alkylating the respective thiocompounds **1a–e, 4** and **6** with 1-adamantyl-methyl or -ethyl bromide in alcoholic KOH solution. Flash column chromatography was used for purification of the products, and the constitution of the products was confirmed by elemental analyses, and by ^{1}H NMR and mass spectra. The compounds prepared are shown in Scheme 1.

All new compounds were tested for their ability to potentiate TNF-α production using the TNF-α gene-transduced clone of B78-H1 murine melanoma cells that has been termed B78/TNF/9. This clone constitutively secretes TNF-α and therefore is useful for testing the effects of various chemicals on TNF-α production [15]. The experiments were performed using the candidate compounds at three concentrations (1 μM, 10 μM and 100 μM). At the highest concentration, the majority of tested compounds exerted a toxic effect that manifested itself in morphological changes in the test cells and was confirmed by the results of the MTT assay. In particular, no living cells were found after 24 h exposure to 100 μM concentrations of compounds **3d** and **7d**. On the other hand, the adamantyl derivatives **2a**, **3e** and **5a** did not show any toxic effects even at this concentration.

Some adamantylated heterocycles, especially **2e**, **5b**, **7a** and **7b**, showed measurable stimulation of

Compound	TNF-α stimulatory activity[a] at			Toxicity at	Stimulatory activity ratio[b] at 10 μM	
	1 μM	10 μM	100 μM	100 μM	Compounds tested	Ratio
2a	86	167	156	0	**2a**/**2as**	0.70
2b	133	226	44	++	**2b**/**2bs**	0.78
2c	106	178	4	+++	**2c**/**2cs**	1.19
2d	128	281	8	+++	**2d**/**2ds**	1.63
2e	154	220	72	++	**2e**/**2es**	1.38
3a	121	192	28	++	**3a**/**2as**	0.81
3b	130	234	29	++	**3b**/**2bs**	0.81
3c	102	155	7	+++	**3c**/**2cs**	1.03
3d	108	173	0	+++	**3d**/**2ds**	1.01
3e	116	164	180	0	**3e**/**2es**	0.96
5a	108	135	250	0	**5a**/**5s**	0.65
5b	145	249	122	+	**5b**/**5s**	1.20
7a	168	164	43	++	**7a**/**7s**	0.80
7b	155	215	0	+++	**7b**/**7s**	1.03
AdAmP[c]	186	308	188	+	—	—

Table 1. TNF-α production-stimulatory activity and cytotoxicity of the tested compounds in the B78/TNF/9 murine melanoma cell line[a].

[a] TNF-α stimulatory activity of the tested compounds was expressed as percent of TNF-α concentration assessed in control cultures (with the addition of DMSO alone: 2350 $\pm$ 235 pg/ml, mean $\pm$SD, $n = 5$). All values shown were means of three independent measurements; standard deviation (SD) are not shown; [b] the ratio of the TNF-α production-stimulatory activity of the tested compound to the TNF-α production-stimulatory activity of its adamantylthio congener; [c] the data for this compound were taken from Ref. [10].

TNF-α secretion at 1 μM concentration. Derivative **2d** was found to be the most active in this respect among the newly synthesized compounds, and was only slightly less active at 10 μM than AdAmP, the most potent enhancer of TNF-α production among the heterocyclic compounds ever synthesized and tested in our laboratory. Having in mind the assumption that the flexibility of the spacer between the heterocyclic core and adamantyl residue is necessary for the biological activity of these compounds we also investigated the influence of additional methylene and ethylene bridges on the TNF-α secretion-stimulating activity. To explore this effect we have been comparing the activity of the adamantylalkylthio derivatives with that of their congener adamantylthio compounds **2as**–**2es** (*e.g.* 2-(1-adamantyl)methylthiopyridine **2a** versus 2-(1-adamantyl)thiopyridine **2as**) (Table 1). In most pyridine derivatives investigated (**2a**, **2b**, **3a** and **3b**), the additional methylene or ethylene link reduced the TNF-α secretion-stimulating potency. A more complex effect was found for pyrimidine derivatives. In this case, introduction of the methylene link enhanced the stimulatory effect on TNF-α secretion (see Table 1, compounds **2c**–**e**), whereas adding of the further methylene link (see compounds **3c**–**e**) reversed this effect and rendered the product's potency similar to that of the corresponding adamantylthiopyrimidine. However, the adamantylethylene derivatives (**5b** and **7b**) of the smaller, five-membered heterocyclic compounds **4** and **6** were more potent then their adamantylmethyl (**5a** and **7a**, respectively) and simple adamantyl congeners (**5s** and **7s**).

The crystal and molecular structures of TNF-α receptor(s) are unknown. We hope that our results presented in this and previously published papers will be helpful in gaining more insight into the structure of these important molecules. TNF-α inducers and enhancers can induce antitumor immune responses and thus carry a considerable therapeutic potential [16]. It has been demonstrated in many recent studies that genetic modification of tumor cells with genes coding for cytokines (including TNF-α) can enhance immunogenicity of these cells [15, 17]. When used as vaccines, cytokine-secreting tumor cells may activate effector mechanisms resulting in tumor regression [15,18]. The efficacy of these mechanisms and immunogenic potential of tumor vaccines is strictly dependent on the amount of cytokines produced by genetically modified tumor cells [19]. We hope that the newly synthesized compounds presented above, due to their TNF-α production-enhancing effect, could help making tumor vaccines more immunogenic and thus might find an application in gene therapy of cancer.

Experimental Section

Instrumentation: All chemicals and solvents were purchased from Sigma-Aldrich. Melting points (uncorr.) were measured in open capillary tubes on a Gallenkamp-5 melting point apparatus. ^{1}H NMR spectra (in ppm) were measured with Varian Gemini 200 MHz and Varian UNITY-plus spectrometers at 298 K in [D_6]-DMSO. Mass spectra (70 eV) were obtained with an AMD-604 (Intectra) spectrometer. Flash chromatography was performed with Merck silica gel (230–400 mesh). Analytical thin layer chromatography (TLC) was carried out on precoated silica gel F_{254} (Merck) plates (0.25 mm thickness).

Synthesis: All the chemicals used were analytical grade commercial products and were used with no further purification.

1-Bromomethyladamantane and 1-bromoethyladamantane. These substrates were obtained from adamant-1-ylmethanol (adamantanemethanol) and 2-(adamant-1-yl)ethanol (adamantaneethanol), respectively, according to the method published previously, and their melting points were the same as those given in the literature [20].

The general procedure: Appropriate mercapto derivatives **1a-e, 4, 6** (2 mmol), 112 mg (2 mmol) of KOH and 300 mg of NaI were dissolved in 10 ml of anhydrous ethanol. Then 2 mmol of 1-bromomethyladamantane or 1-bromoethyladamantane were added. The mixture was stirred at room temperature for 6 h. After this time ethanol was evaporated. The crude products were purified by flash chromatography (SiO_2) using a hexane/ethyl acetate (5:1) mixture.

2-(Adamantan-1-ylmethylsulfanyl)-pyridine **(2a)**: 650 mg (62.7%). M.p. 33 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 1.55 – 1.93 (m, 15 H, adamant.), 3.05 (s, 2 H, S*CH*$_2$), 7.06 (m, 1H, pyr.), 7.27 (m, 1H, pyr.), 7.58 (m, 1H, pyr.), 8.40 (m, 1H, pyr.). – MS (EI, 70 eV): *m/z* (%) = 259 (34) [M^+], 135 (100) [Ada-], 124 (14) [M^+-AdaCH_2S]. – $C_{16}H_{21}NS$ (259.4): calcd. C 74.08, H 8.16, N 5.40; found C 74.01, H 8.20, N 5.24.

2-(Adamantan-1-ylmethylsulfanyl)-6-methylpyridine **(2b)**: Colourless oil (340 mg, 62.3%). – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.50 - 1.93$ (m, 15 H, adamant.), 2.41 (s, 3 H, *Me*), 3.02 (s, 2 H, S*CH*$_2$), 6.92 (d, $J = 7.7$ Hz, 1 H, pyr.), 7.08 (d, $J = 7.7$ Hz, 1 H, pyr.), 7.48 (t, $J = 7.7$ Hz, 1 H, 4-H). – MS (EI, 70 eV): *m/z* (%) = 273 (78) [M^+], 138 (29) [M^+-Ada].- $C_{17}H_{23}NS$ (273.4): calcd. C 74.67, H 8.48, N 5.12; found C 74.61, H 8.44, N 5.04.

2-(Adamantan-1-ylmethylsulfanyl)-pyrimidine **(2c)**: (300 mg, 28.8%). M.p. 73 – 75 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.55 - 1.94$ (m, 15 H, adamant.), 3.05 (s, 2 H, S*CH*$_2$), 7.17 (t, $J = 4.9$ Hz, 1 H, 5-H), 8.60 (d, $J = 4.9$ Hz, 2 H, 4-H, 6-H). – MS (EI, 70 eV): *m/z* (%) = 260 (57) [M^+], 227 (93), 135 (100) [Ada-], 93 (24), 79 (26). – $C_{15}H_{20}N_2S$ (260.4): calcd. C 69.19, H 7.74, N 10.76; found C 69.29, H 7.80, N 10.81.

2-(Adamantan-1-ylmethylsulfanyl)-4-methylpyrimidine **(2d)**: (320 mg, 32%). M.p. 57 – 59 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.54 - 1.93$ (m, 15 H, adamant.), 3.04 (s, 2 H, S*CH*$_2$), 7.04 (d, $J = 7.0$ Hz, 1 H, 5-H), 8.43 (d, $J = 7.0$ Hz, 1 H, 6-H). – MS (EI, 70 eV): *m/z* (%) = 274 (29) [M^+], 241 (100), 135 (60) [Ada-], 93 (17), 79 (17). – $C_{16}H_{22}N_2S$ (274.4): calcd. C 70.03, H 8.08, N 10.21; found C 70.00, H 8.15, N 10.14.

2-(Adamantan-1-ylmethylsulfanyl)-4,6-dimethylpyrimidine **(2e)**: (490 mg, 42.5%). M.p. 68 – 71 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.54 - 1.93$ (m, 15 H, adamant.), 2.33 (s, 6 H, *Me*) 3.03 (s, 2 H, S*CH*$_2$), 6.91 (s, 1 H, 5-H). – MS (EI, 70 eV): *m/z* (%) = 288 (18) [M^+], 255 (100), 153 (12) [M^+-AdaCH_2S], 135 (33) [Ada-]. – $C_{17}H_{24}N_2S$ (288.5): calcd. C 70.79, H 8.39, N 9.71; found C 70.92, H 8.29, N 9.59.

2-(2-Adamantan-1-yl-ethylsulfanyl)-pyridine **(3a)**: Colourless oil (490 mg, 89.7%). – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.38$ (m, 2 H, *CH*$_2$), 1.50 – 1.91 (m, 15 H, adamant.), 3.06 (m, 2 H, S*CH*$_2$), 7.05 (m, 1H, pyr.), 7.20 (m, 1s, pyr.), 7.54 (m, 1H, pyr.), 8.40 (m, 1H, pyr.). – MS (EI, 70 eV): *m/z* (%) = 273 (61) [M^+], 240 (96), 213 (25), 138 (100) [M^+-Ada], 111 (49). – $C_{17}H_{23}NS$ (273.4): calcd. C 74.67, H 8.48, N 5.12; found C 74.61, H 8.44, N 5.03.

2-(2-Adamantan-1-yl-ethylsulfanyl)-6-methylpyridine **(3b)**: Colourless oil (460 mg, 80.1%). – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.37$ (m, 2 H, *CH*$_2$), 1.51 – 1.93 (m, 15 H, adamant.), 2.41 (s, 3 H, *Me*), 3.04 (m, 2 H, S*CH*$_2$), 6.93 (d, $J = 7.7$ Hz, 1 H, pyr.), 7.02 (d, $J = 7.7$ Hz, 1 H, pyr.), 7.50 (t, $J = 7.7$ Hz, 1 H, 4-H). – MS (EI, 70 eV): *m/z* (%) = 287 (1) [M^+], 254 (4), 163 (21), 135 (100) [Ada-]. – $C_{18}H_{25}NS$ (287.5): calcd. C 75.21, H 8.77, N 4.87; found C 75.11, H 8.80, N 4.72.

2-(2-Adamantan-1-yl-ethylsulfanyl)-pyrimidine **(3c):** (480 mg, 87.6%). M.p. 72 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.41$ (m, 2 H, *CH*$_2$), 1.50 – 1.92 (m, 15 H, adamant.), 3.05 (m, 2 H, S*CH*$_2$), 7.17 (t, $J = 4.8$ Hz, 1 H, 5-H), 8.60 (d, $J = 4.8$ Hz, 2 H, 4-H, 6-H). – MS (EI, 70 eV): *m/z* (%) = 274 (29) [M^+], 241 (100), 139 (32) [M^+-Ada], 135 (16) [Ada-], 112 (73). – $C_{16}H_{22}N_2S$ (274.4): calcd. C 70.03, H 8.08, N 10.21; found C 70.01, H 8.00, N 10.03.

2-(2-Adamantan-1-yl-ethylsulfanyl)-4-methylpyrimidine **(3d).** (430 mg, 74.7%). M.p. 55 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.41$ (m, 2 H, *CH*$_2$), 1.52 – 1.92 (m, 15 H, adamant.), 3.03 (m, 2 H, S*CH*$_2$), 7.03 (d, $J = 5.0$ Hz, 1 H, 5-H), 8.43 (d, $J = 5.0$ Hz, 1 H, 6-H). – MS (EI, 70 eV): *m/z* (%) = 288 (53) [M^+], 255 (100), 153 (63) [M^+-Ada], 127 (27), 126 (95).- $C_{17}H_{24}N_2S$ (288.5): calcd. C 70.79, H 8.39, N 9.71; found C 70.71, H 8.33, N 9.84.

2-(2-Adamantan-1-yl-ethylsulfanyl)-4,6-dimethylpyrimidine **(3e):** (525 mg, 86.9%). M. p. 95 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.40$ (m, 2H, *CH*$_2$), 1.50-1.93 (m, 15 H, adamant.), 2.37 (s, 6H, *CH*$_3$), 3.05 (m, 2H, S*CH*$_2$), 6.92 (s, 1H). – MS (EI, 70 eV): *m/z* (%) = 302 (35) [M^+], 269 (100), 167 (47) [M^+-Ada], 140 (30). – $C_{18}H_{26}N_2S$ (302.5): calcd. C 71.47, H 8.66, N 9.26; found C 71.55, H 8.70, N 9.13.

2-(Adamantan-1-ylmethylsulfanyl)-4,5-dihydrothiazole **(5a):** Colourless oil (280 mg, 26.2%). – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.51 - 1.94$ (m, 15 H, adamant.), 3.05 (s, 2 H, S*CH*$_2$), 3.49 (t, $J = 8.0$ Hz, 2 H, thiazole), 4.14 (t, $J = 8.0$ Hz, 2 H, thiazole). – MS (EI, 70 eV): *m/z* (%) = 267 (100) [M^+], 234 (99), 220 (21), 208 (22), 135 (47) [Ada-], 93 (31), 79 (26).- $C_{14}H_{21}NS_2$ (267.5): calcd. C 62.87, H 7.91, N 5.24; found C 62.99, H 8.03, N 5.04.

2-(2-Adamantan-1-yl-ethylsulfanyl)-4,5-dihydrothiazole **(5b):** (495 mg, 88.1%). M. p. 67 °C. – ^{1}H NMR (200 MHz,

$CDCl_3$): $\delta = 1.40$ (m, 2 H, CH_2), 1.47-1.92 (m, 15 H, adamant.), 3.03 (m, 2 H, SCH_2), 3.41 (t, $J = 7.9$ Hz, 2 H, thiazole), 4.13 (t, $J = 7.9$ Hz, 2 H, thiazole). – MS (EI, 70 eV): *m/z* (%) = 281 (25) [M^+], 248 (13), 146 (13) [M^+-Ada], 135 (13) [Ada-], 119 (100), 114 (29). – $C_{15}H_{23}NS_2$ (281.5): calcd. C 64.01, H 8.24, N 4.98; found C 64.11, H 8.18, N 4.84.

*2-(Adamantan-1-ylmethylsulfanyl)-1-methyl-1*H-*imidazole* **(7a):** (300 mg, 28.6%). M.p. 64 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.51-1.93$ (m, 15 H, adamant.), 2.91 (s, 2 H, SCH_2), 3.57 (s, 3 H, N*Me*), 6.89 (m, 1 H, imidazole), 7.17 (m, 1 H, imidazole). – MS (EI, 70 eV): *m/z* (%) = 262 (10) [M^+], 229 (100), 135 (8) [Ada-], 114 (18). – $C_{15}H_{22}N_2S$ (262.4): calcd. C 68.66, H 8.45, N 10.68; found C 68.76, H 8.40, N 10.55.

*2-(2-Adamantan-1-yl-ethylsulfanyl)-1-methyl-1*H-*imidazole* **7b):** (405 mg, 73.4%). M.p. 68 °C. -^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.35$ (m, 2 H, CH_2) 1.44-1.91 (m, 15 H, adamant.), 2.93 (m, 2 H, SCH_2), 3.56 (s, 3 H, N*Me*), 6.92 (m, 1 H, imidazole), 7.20 (m, 1 H, imidazole). – MS (EI, 70 eV): *m/z* (%) = 276 (15) [M^+], 243 (24), 141 (17) [M^+-Ada], 114 (100), 109 (48). – $C_{16}H_{24}N_2S$ (276.5): calcd. C 69.52, H 8.75, N 10.13; found C 69.66, H 8.71, N 10.02.

TNF-α assessment in cell cultures: The ability of the compounds described above to stimulate TNF-α production was studied in B78-H1 murine melanoma cells that have been transduced with the gene for human TNF-α (clone 9, termed B78/TNF/9) [15]. This clone secretes TNF-α constitutively and therefore is useful for testing the effect of various chemicals on TNF-α production. B78/TNF/9 melanoma cells (2×10^5 cells/ml) were incubated with the investigated compounds for 24 h. The concentration of TNF-α in culture supernatants was measured using enzyme-linked immunosorbent assay (ELISA). For comparison, the respective data for AdAmP, which compound was found to be one of the most potent enhancers of TNF-α production [10], were also included in Table 1.

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