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Synthesis of N-Substituted 3,5-Bis(arylidene)-4-piperidones with High Antitumor and Antioxidant Activity

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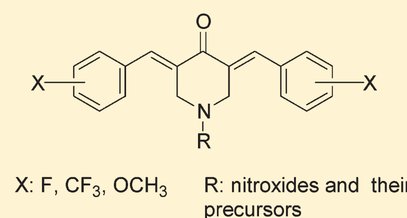
Supporting Information

ABSTRACT: A series of 3,5-bis(arylidene)-4-piperidone (DAP) compounds are considered as synthetic analogues of curcumin for anticancer properties. We performed structure—activity relationship studies by synthesizing a number of DAPs N-alkylated or acylated with nitroxides or their amine precursors as potent antioxidant moieties. Both subtituents on arylidene rings and on piperidone nitrogen (five- or six-membered, 2- or 3-substituted or 3,4-disubstituted isoindoline nitroxides) were varied. The anticancer efficacy of the new DAP compounds was tested by measuring their cytotoxicity to cancer cell lines A2780 and MCF-7 and to the H9c2 cell line. The results showed that all DAP compounds induced a significant loss of cell viability in the human cancer cell lines tested; however, only pyrroline appended nitroxides (**5c** (Selvendiran, K.; Tong, L.; Bratasz, A.; Kuppusamy, L. M.; Ahmed, S.; Ravi, Y.; Trigg, N. J.; Rivera, B. K.; Kálai, T.; Hideg, K.; Kuppusamy, P. Mol. Cancer Ther. 2010, 9, 1169—1179), **5e**, **7**, **9**) showed limited toxicity toward noncancerous cell lines. Computer docking simulations support the biological activity tested. These results suggest that antioxidant-conjugated DAPs will be useful as a safe and effective anticancer agent for cancer therapy.



INTRODUCTION

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)1,6-heptadien-3,5-dion), a natural component of the rhizome of *Curcuma longa*, proved to be a powerful chemopreventive and anticancer agent[2−5] having also anti-inflammatory,[6] antibacterial,[7] and antioxidant properties.[8] However, the clinical use of curcumin has been limited because of its low anticancer activity and poor bioabsorption. In the past decade, a novel class of curcumin analogues, DAPs, has been developed by incorporating a piperidone link to the β-diketone structure and fluoro-, methoxy-, hydroxyl-, chloro-, nitro-, dimethylamino substituents on the phenyl group.[9,10] These curcumin analogues exhibited multidrug-resistance reverting[11,12] and antimycobacterial[13] properties as well. The idea of evaluation of these compounds as antineoplastic agents is based on the assumption that these compounds may be considered as a Mannich base of dienone and α,β-unsaturated ketones. These kinds of compound display anticancer properties via a mechanism of action comprising interactions with cellular thiols with little or no affinity for hydroxyl and amino groups in nucleic acids. The 1,5-diaryl-3-oxo-1,4-pentadienyl groups are considered to react at a primary binding site; however, the bioactivity will be influenced by other structural units as well. The acylation of piperidone nitrogen increased the cytotoxic potencies, and increasing the electron-withdrawing properties of substituents on aromatic ring has an advantageous effect on cytotoxicity.[14−18] The dimethylene bridge between C2 and C6 atoms in piperidone was accompanied by reduction of cytotoxic properties exerting a steric impedance to alignment at one or more binding sites as well as variation of hydrophobicity and hence membrane transportation properties.[16]

In general the DAP compounds were more effective than curcumin in inhibiting the proliferation of a variety of cancer cell lines. For example 3,5-bis[2-(fluoro)benzylidene]piperidin-4-one (EF24) with ortho-fluorinated phenyl group exhibited anticancer activity in vitro when tested using breast cancer, colon cancer, and ovarian epithelial cancer.[19−21] Its para-fluorinated derivative 3,5-bis[4-(fluoro)benzylidene]piperidin-4-one (H-4073) was more potent than **6** (EF24) in inducing cytotoxicity to ovarian cancer cells.[21−23] DAP compounds have also been shown to be more readily bioavailable than the parent compound curcumin.[24]

A nonspecific cytotoxic compound may have side effects caused by damage to normal cells. Many chemotherapeutic agents act by producing free radicals, causing oxidative stress in normal cells.[25] It is well-known that nitroxides or their precursors (hydroxylamines and sterically hindered amines) scavenge oxygen radicals in cells that have normal redox status and have beneficial effect on toxicity and/or efficiency in reactive oxyges species (ROS) scavenging compared to the original drug.[26,27] Our previous studies indicated that nitroxides or their amine precursors play multiple roles in elimination of ROS formed during doxorubicine metabolism without reducing their anticancer effect.[28,29] These results inspired us to combine anticancer and antioxidant properties to decrease ROS-promoted damage. The DAPs were ideal

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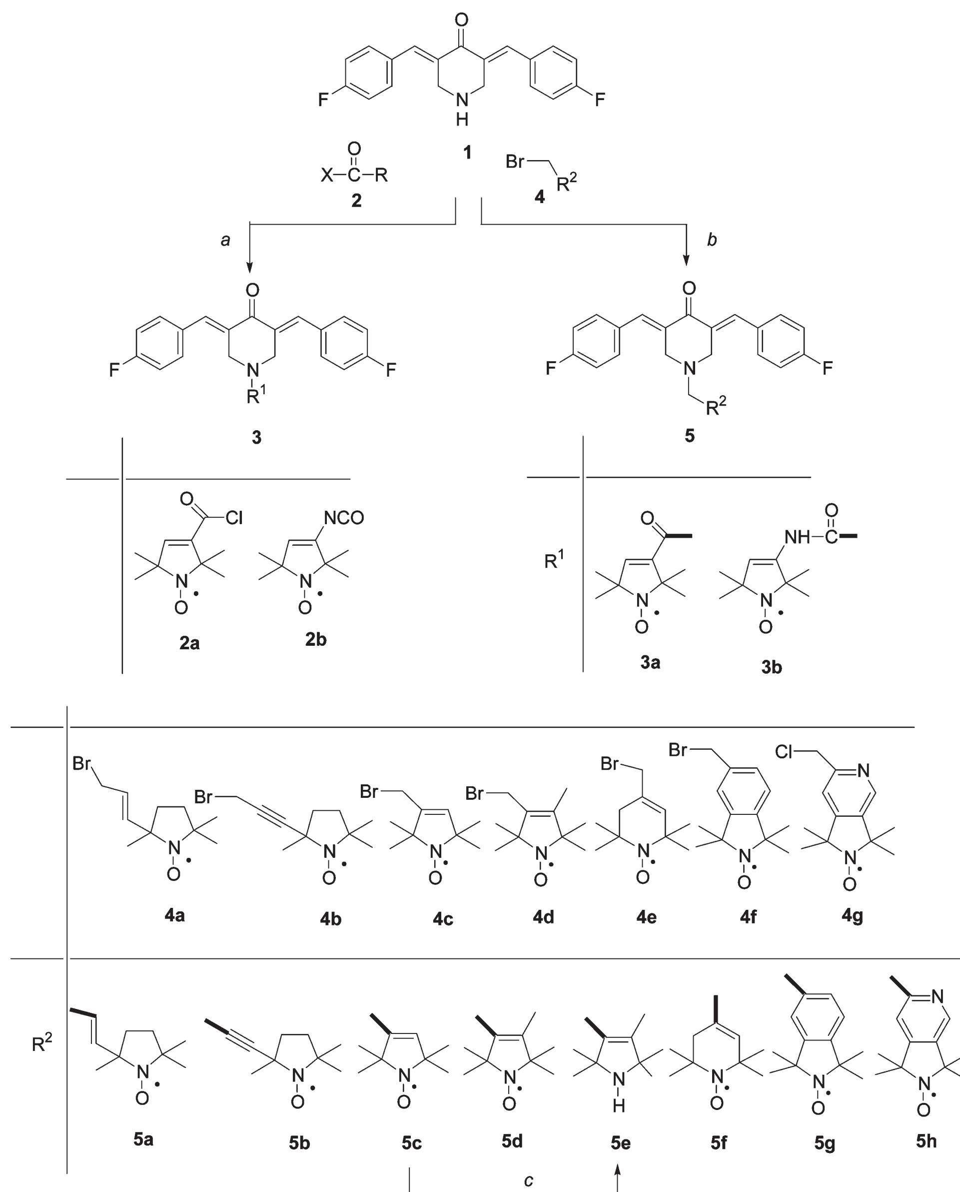
Scheme 1[a]



[a] Reagents and conditions: (a) compound **2a** (1.0 equiv), Et_3N (2.0 equiv) CH_2Cl_2, 0° C → room temp, 1 h, 49%, or compound **2b** (1.0 equiv), THF, reflux, 4 h, 62%; (b) **4a**−**h** (1.0 equiv), K_2CO_3, (1.0 equiv), acetonitrile, reflux, 3 h, 35−68%; (c) Fe (10.0 equiv), AcOH, 60 °C, 35 min, 56%.

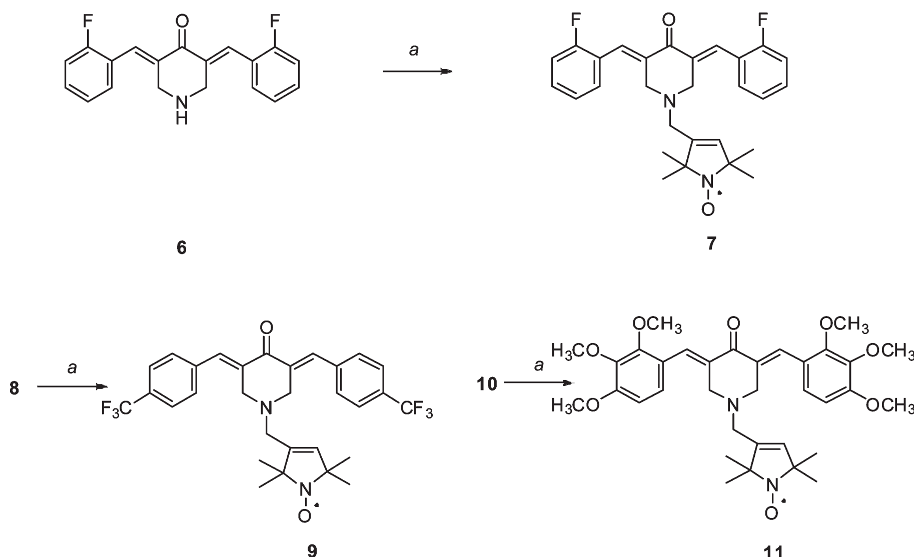
candidates to prove the conception because of the easily variable nitrogen substituents of the piperidone moiety. This study presents the synthesis and evaluation of new DAP compounds with different substituents (F, CF_3, OCH_3) on the aromatic rings, as well as variation of nitroxides (saturated, unsaturated, six-membered, isoindoline, etc.) attached to the piperidone nitrogen.

The study showed that the DAPs induce preferential toxicity in cancer cells while sparing noncancereous cells. The results suggest that the antioxidant (nitroxide) conjugated DAPs will be useful as safe and effective anticancer agents for cancer therapy.

■ CHEMISTRY

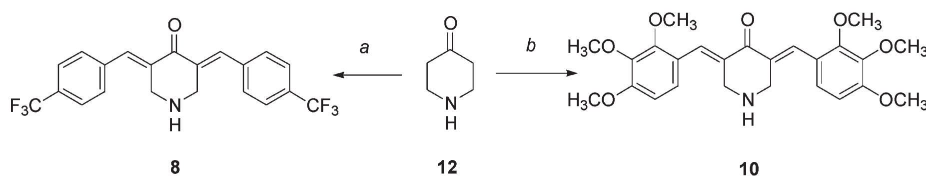
A Claisen−Schmidt condensation between 4-piperidone hydrochloride and the appropriate aldehyde led to the formation of DAPs. On the basis of earlier X-ray crystallography data, we propose that compounds **1** (H-4073), **6**, **8**, **10** possess *E* stereochemistry.[10,14,15] The new *N*-acyl-3,5-bis(4-fluorobenzylidene)-piperidin-4-ones were prepared by treatment of compound **1** with freshly prepared paramagnetic acyl chloride **2a**[30] in the presence of Et_3N in CH_2Cl_2 or treatment with isocyanate **2b**[31] generated in situ by Curtius rearrangement of acyl azide in THF to yield compounds **3a** and **3b**. The *N*-alkyl 3,5-bis(4-fluorobenzylidene)-piperidin-4-ones were achieved by alkylation of compound **1** with an equivalent amount of paramagnetic alkyl halides **4a**,[32] **4b**,[32] **4c**,[33] **4d**,[34] **4e**,[35] **4f**,[36] **4g**[37] in acetonitrile in the presence of K_2CO_3 to give compounds **5a**, **5b**, **5c**, **5d**, **5f**, **5g**, **5h**. Compound **5e** was synthesized by reduction of the nitroxide function of compound **5c** with iron powder in glacial acetic acid[38] (Scheme 1). Alkylation of 3,5-bis(2-fluorobenzylidene)piperidin-4-one **6**,[19] 3,5-bis(4-trifluoromethylbenzylidene)piperidin-4-one **8**, and

Scheme 2[a]



[a] Reagents and conditions: (a) compound **4c** (1.0 equiv), K_2CO_3, (1.0 equiv), acetonitrile, reflux, 3 h, 55−71%.

Scheme 3[a]



[a] Reagents and conditions: (a) 4-trifluoromethylbenzaldehyde (2.0 equiv), AcOH saturated with HCl gas, 48 h, room temp, 74%; (b) 2,3,4-trimethoxybenzaldehyde (2.0 equiv), AcOH saturated with HCl gas, 48 h, room temp, 62%.

3,5-bis(2,3,4-trimethoxybenzylidene)piperidin-4-one **10** with compound **4c** as above yielded compounds **7**, **9**, **11**, respectively (Scheme 2). Compounds **8** and **10** were synthesized by condensation of piperidine-4-one HCl (**12**) salt with 4-trifluoromethylbenzaldehyde or 2,3,4-trimethoxybenzaldehyde in AcOH saturated previously with HCl gas (Scheme 3).

RESULTS AND DISCUSSION

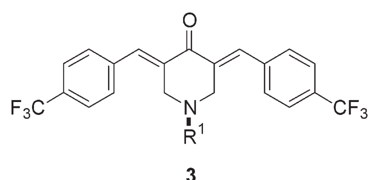
The anticancer efficacy of the DAP compounds with various substituents on aromatic rings and on piperidone nitrogen was evaluated by measuring the cytotoxicity of the compounds to well-established cancer cell lines, namely, A2780 and MCF-7 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The measurements were performed by exposing the cells to 10 μM compound for 24 h. The results, in the form of percent cell viability compared to respective control, are summarized in Tables 1−3. The results showed that all DAP compounds induced a significant loss of cell viability in both of the human cancer cell lines tested. In particular 3,5-bis(arylidene)-4-piperidone compounds without nitroxide tag (**1**, **6**, **8**, **10**) demomstrated a substantial cytotoxic effect against A2780 and MCF-7 cells. The electron-withdrawing substituents (F, CF_3) containing derivatives (**1**, **6**, **8**) exhibited greater cytotoxicity than trimethoxy derivative **10**, in agreement with previous findings.[14,15] In all caseses the toxicity can be increased by modifying the DAP compounds with nitroxides by acylation (**3a**, **3b**) and by alkylation (**5a**−**h**, **7**, **9**, **11**). In particular **3a** and **3b** demonstrated a substantial cytotoxic effect against A2780 and MCF-7 cells. Comparable cytotoxic efficacies were observed with **5c**, **5e**, **5f**, and **9** derivatives. The results further indicated that the DAPs were more cytotoxic to ovarian cancer cell (A2780) than to breast cancer cell. Compounds containing 2-substituted pyrrolidine nitroxide (**5a**, **5b**), 3,4-disubstituted nitroxide (**5d**), or isoindoline-type nitroxides (**5g**, **5h**) exhibited limited toxicity toward breast cancer cell lines.

We also compared the cytotoxicity of DAPs to a noncancerous (healthy) cardiac cell line, namely, H9c2, an undifferentiated neonatal rat cardiomyoblast. Most of the compounds induced a significant loss of cell viability, although to different extents (Table 1−3), the pyrroline-appended DAPs **5c**, **5e**, and **7** were significantly less toxic to the healthy cell.

Particularly, the results of **5c** seem to suggest a strikingly differential effect on cancer versus noncancerous cells. Compound **6**, which was toxic to breast cancer cell, was toxic to healthy cells to the same extent. In addition, this differential effect could stem from the *N*-hydroxypyrroline function. Overall the viability results seem to implicate the diarylidenylpiperidone group in inducing cytotoxicity and *N*-hydroxypyrroline group in protecting noncancerous cells.

We recently reported the anticancer efficacy of four DAPs, namely, **1** and **8** without NOH function and **5c** and **9** with NOH

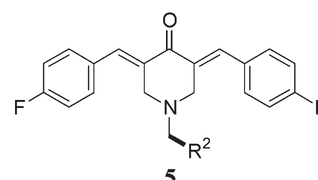
Table 1. Biological Activity of New *N*-Acyl-3,5-bis(4-fluorobenzylidene)piperidin-4-ones



Compound	R[1]	A2780 Viability (%)*	MCF-7 Viability (%)	H9c2 Viability (%)
1 H-4073	H	12.40± 2.62	17.14±1.98	61.40±13.74
3a HO-4049		4.54±0.66	12.42± 2.70	33.29± 5.58
3b HO-4060		5.90±1.57	12.64±1.41	36.70± 6.73

*Control is 100 %.

Table 2. Biological Activity of *N*-Alkyl-3,5-bis(4-fluorobenzylidene)piperidin-4-ones

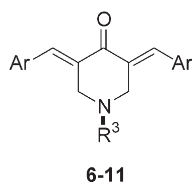


Compound	R[2]	A2780 Viability (%)	MCF-7 Viability (%)	H9c2 Viability (%)
5a HO-4151		5.81±1.25	43.15±6.30	51.90± 10.08
5b HO-4147		5.89±1.36	38.34±3.81	50.66± 8.72
5c HO-3867		20.48±4.60	16.56±3.69	88.50± 13.25
5d HO-4146		6.75±0.64	41.98±4.59	56.79±6.74
5e HO-3868		4.79±0.55	28.28±0.90	78.83± 10.47
5f HO-4059		6.86±1.05	20.12±4.83	51.36±4.81
5g HO-4104		9.50±3.71	46.21±5.63	26.35±4.81
5h HO-4180		5.26±0.86	42.71±5.60	46.57±7.40

function against a number of cancerous (breast, colon, head and neck, liver, lung, ovarian, and prostate cancer) and noncancerous (smooth muscle, aortic endothelial, and ovarian surface epithelial cells) human cell lines. We observed that all four compounds induced significant loss of cell viability in cancer cells, while **5c** and **9** showed significantly less cytotoxicity in noncancerous (healthy) cells. Electron paramagnetic resonance (EPR) measurements showed a metabolic conversion of the *N*-hydroxylamine function to nitroxide with significantly higher levels of the metabolite and superoxide radical-scavenging (antioxidant) activity in noncancerous cells when compared to cancer cells. The antioxidant activity of compounds **5c** and **9** in comparison to their base (non-nitroxide-derivatized) compounds **1** and **8** was reported recently.[22] EPR measurements demonstrated substantial ability of **5c** and **9** to scavenge superoxide radicals but not the base compounds **1** and **8**. Among the new compounds **5c** exhibited the best selective toxicity against cancerous cell. For this reason, this compound was chosen as a new lead compound for further evaluations. Western blot analysis showed that the DAP-induced growth arrest and apoptosis in cancer cells were mediated by inhibition of STAT3 phosphorylation at Tyr705 and Ser727 residues and induction of apoptotic markers of cleaved caspase-3 and poly ADP-ribose polymerase (PARP), suggesting that the antioxidant-conjugated DAPs will be useful as a safe and effective anticancer agent for cancer therapy.[22] In a subsequent study, we further confirmed the anticancer efficacy of **5c** in a number of established human ovarian cancer cell lines (A2870, A2780cDDP, OV-4, SKOV3, PA-1, and OVCAR3), as well as in a murine xenograft tumor (A2780) model.[1] Compound **5c** demonstrated a preferential toxicity toward ovarian cancer cells while sparing healthy cells. It induced G2/M cell-cycle arrest in A2780 cells by modulating cell-cycle regulatory molecules p53, p21, p27, cdk2, and cyclin, and promoted apoptosis by caspase-8 and caspase-3 activation. It also caused an increase in the expression of functional Fas/CD95 and decreases in STAT3 (Tyr705) and JAK1 phosphorylation. There was a significant reduction in STAT3 downstream target protein levels including Bcl-xL, Bcl-2, survivin, and vascular endothelial growth factor (VEGF), suggesting that **5c** exposure disrupted the JAK/STAT3-signaling pathway. In addition, compound **5c** significantly inhibited the growth of the ovarian xenografted tumors in a dosage-dependent manner without any apparent toxicity. Western blot analysis of the xenograft tumor tissues showed that compound **5c** inhibited pSTAT3 tyrosine 705 and serine 727 (Tyr705 and Ser727) and JAK1 and increased apoptotic markers cleaved caspase-3 and PARP. Overall, compound **5c** exhibited significant cytotoxicity toward ovarian cancer cells by inhibition of the JAK/STAT3-signaling pathway.[1]

The cellular uptake of **5c** was measured in a variety of cancer cell lines. Compound **5c** was taken in cells within 15 min of exposure, and its uptake was more than 100-fold higher than that of curcumin. Compound **5c** was also retained in cells in an active

Table 3. Biological Activity of *N*-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-ylmethyl)-3,5-bis(4-arylidene)piperidin-4-ones



6-11

Compound	Ar	R^3	A2780 Viability (%)	MCF-7 Viability (%)	H9c2 Viability (%)
6 L-2359	*o*-F-Ph	H	12.79± 2.65	34.55± 2.26	32.88± 6.22
7 HO-3865	*o*-F-Ph		12.48± 1.14	53.50± 8.36	86.35± 7.82
8 H-4138	*p*-CF_3-Ph	H	13.10± 0.46	20.19± 2.49	51.74±12.10
9 HO-4200	*p*-CF_3-Ph		5.85±0.63	14.23±1.71	17.06± 0.86
10 H-4139	2.3.4-MeO-Ph	H	10.99± 1.10	56.56± 6.18	32.26± 5.43
11 HO-4196	2.3.4-MeO-Ph		26.07± 4.43	27.96± 1.94	53.13± 7.14

Table 4. Summary of DAP Compound Docking Simulations with the Murine STAT3 Dimer (PDB Code 3CWG)

simulation test compd	lowest five binding energies (kcal/mol)	corresponding constant of inhibition K_i (nmol)
1	−8.76[a]	380.28
	−8.76[a]	379.04
	−8.76[a]	378.52
	−8.76[a]	379.50
	−8.75	386.19
8	−7.89[a]	1660
	−7.89[a]	1660
	−7.88	1670
	−7.87[a]	1710
	−7.87[a]	1700
5c	−11.15[a]	6.75
	−11.15[a]	6.76
	−11.10	7.24
	−11.00	8.67
	−10.99	8.80
9	−10.66	15.33
	−10.62	16.32
	−10.61	16.68
	−10.59[a]	17.22
	−10.59[a]	17.34

[a] The similarity of docked structures is measured by computing the root-mean-square deviation (rmsd) between the coordinates of the atoms and creating a clustering of the conformations based on these rmsd values. In this run, multiple binding conformation clusters were found, some with equivalent binding energies.

form for 72 h and possibly longer. When administered to rats by intraperitoneal injection, significantly high levels of **5c** were found in the liver, kidney, stomach, and blood after 3 h. Also, significant accumulation of **5c** was found in murine tumor xenografts with a dose-dependent inhibition of tumor growth. The results suggest that **5c** has substantially higher bioabsorption when compared to curcumin.[24] The advantageous effect of nitrogen substitution of DAPs is also demonstrated by results of Youssef et al. They reported that amino acid conjugates have better cytostatic activity and bioavailability than DAPs without N-substitution.[39]

MOLECULAR DOCKING

We performed theoretical modeling calculations using AutoDock (version 4.2) to elucidate the mode of STAT3-inhibition by these compounds using in silico docking simulations. The simulation results have provided additional support for the hypothesis that the mechanism of action of the DAP compounds is via targeting of the STAT3 pathway. The computations demonstrate that the DAP compounds have high docking affinity for the STAT3 dimer (PDB code 3CWG) at the deoxyribonucleic acid (DNA) binding domain of the molecule (Table 4, Figure 1). This in turn would prevent the activated STAT3 molecule from binding with DNA, thereby inhibiting transcription of downstream signaling. One of the more notable results from the docking simulations is that the DAP compounds with the *N*-hydroxypyrroline (nitroxide) moiety, **5c** and **9**, have substantially lower binding energies than the parent molecules lacking this functional group (**1** and **8**). We believe that this is most likely due to the fact that the molecules bearing the *N*-hydroxylamine group are more polar and have a pronounced asymmetry. Upon examination of the preferred pocket of the DAP compounds within the DNA-binding domain of the STAT3

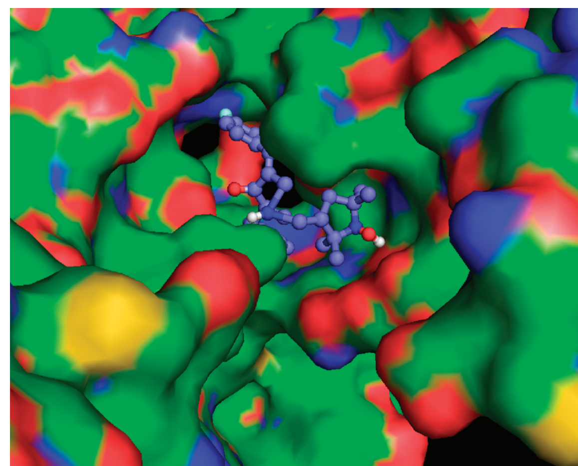


Figure 1. Compound **5c** docked to STAT3 dimer. This image was generated with the free version of PyMol using computational data from the AutoDock simulations.

dimer, it was noted that amino acid residues present include arginine (Arg), glutamic acid (Glu), Tyr, Ser, aspartic acid (Asp), asparagine (Asn), and threonine (Thr). All of these amino acids are classified as polar or as having polar side chains.

Thus, it stands to reason that DAP variants with modifications to increase polarity may display even greater binding affinity (lower binding energy) for the unphosphorylated murine STAT3 molecule at the targeted binding site on the DNA-binding domain. We also note that the molecules with the monofluoro substitution (**1** and **5c**) on the aromatic rings exhibit greater binding affinity than the corresponding trifluoromethyl-substituted compounds (**8** and **9**). We believe that this is due to the size (bulkiness) of the trifluoromethyl group, which acts as an impediment to docking, versus the monofluoro group. Table 4 lists the tested compounds and the five lowest calculated binding energies and the corresponding constants of inhibition (K_i, nmol). The DAP compound binding affinity to the STAT3 dimer determined is ranked as follows: **5c** > **9** ≫ **1** > **8**. The rankings also correlate well with the biological activity. Many small molecules identified as potential drug candidates have reported binding energies in the −7 to −9 kcal/mol range, but few of these potential drug candidates have K_i < 50 nM. In addition, many STAT3 inhibitors target the SH2 binding domain of the molecule, whereas the DAP compounds favor the DNA binding region. The identification of a previously unreported docking site for molecular inhibition of STAT3 activity would be of significant benefit for future drug design.

CONCLUSIONS

The present study demonstrated that the earlier DAP compounds can be N-alkylated or acylated with nitroxides or their amine precursors as potent antioxidant moieties. Measurement of the cytotoxicity of the new compounds to cancer cell lines A2780 and MCF-7 and to H9c2 noncancerous (healthy) cardiac cell line has shown that the modified compounds are more effective as anticancer compounds but at the same time was less toxic to noncancereous (healthy) cells. Computer docking simulations support the empirical data collected. Among the compounds tested **5c** was chosen as the lead compound for further studies. These results support the earlier findings that nitroxides and their precursors do not compromise the anticancer effect of the modifed molecules, but they have a beneficial effect on the original activity.

EXPERIMENTAL SECTION

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on a Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi and VG TRIO-2 instruments and in the EI mode. ^{1}H NMR spectra were recorded with a Varian UNITY INOVA 400 WB spectrometer. Chemical shifts are referenced to Me_4Si. Measurements were run at 298 K probe temperature in $CDCl_3$ solution. ESR spectra were taken on Miniscope MS 200 in 10^{-4} M $CHCl_3$ solution, and monoradicals gave a triplet line. Flash column chromatography was performed on Merck Kieselgel 60 (0.040−0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 cm × 20 cm × 0.02 cm) coated with Merck Kieselgel GF_{254}. All chemicals were purchased from Aldrich. Compounds **1**,[10] **4a**,[32] **4b**,[32] **4c**,[33] **4d**,[34] **4e**,[35] **4f**,[36] **4g**,[37] **5c**,[1] and **6**[19] were prepared as described earlier. All compounds were more than 95% pure.

3,5-Bis(4-fluorobenzylidene)-1-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)carbonyl]piperidin-4-one Radical (3a). To a solution of compound **1** HCl salt (1.73 g, 5.0 mmol) and Et_3N (1.0 g, 10.0 mmol) in CH_2Cl_2 (35 mL), freshly prepared **2a** (1.01 g, 5.0 mmol) dissolved in CH_2Cl_2 (10 mL) was added dropwise at 0 °C. The mixture was allowed to warm to room temperature and stirred for 1 h. The organic phase was washed with brine (20 mL). The organic phase was separated, dried ($MgSO_4$), filtered, and evaporated. The residue was purified by flash column chromatography ($CHCl_3/Et_2O$) to yield the title compound as a yellow solid 1.26 g (49%): mp 168−170 °C, R_f = 0.55 ($CHCl_3/Et_2O$, 2:1). MS (EI) *m/z* (%): 477 (M^+, 6), 463 (15), 447 (10), 310 (30), 133 (100). Anal. Calcd for $C_{28}H_{27}F_2N_2O_3$: C 70.43, H 5.70, N 5.87. Found: C 70.24, H 5.88, N 6.01.

3,5-Bis(4-fluorobenzylidene)-4-oxo-*N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)piperidine-1-carboxamide Radical (3b). To a solution of compound **1** (933 mg, 3.0 mmol) in anhydrous THF (40 mL), compound **2b** (627 mg, 3.0 mmol) was added. The mixture was heated under reflux for 4 h. After the mixture was cooled, the THF was evaporated off under reduced pressure. The residue was partitioned between CH_2Cl_2 (40 mL) and brine (10 mL). The organic phase was washed with 10% aqueous K_2CO_3 (20 mL) and water (10 mL). The organic phase was separated, dried ($MgSO_4$), filtered, and evaporated. The residue was purified by flash column chromatography ($CHCl_3$/MeOH) to yield the title compound as a yellow solid: 915 mg (62%), mp 175−177 °C, R_f = 0.43 ($CHCl^3/Et_2O$, 2:1). MS (EI) *m/z* (%): 492 (M^+, 1), 462 (1), 460 (8), 310 (22), 133 (100). Anal. Calcd for $C_{28}H_{28}F_2N_3O_3$: C 68.28, H 5.73, N 8.53. Found: C 68.12, H 5.90, N 8.68.

General Procedure for N-Alkylation of 3,5-Bis(arylidene)-piperidin-4-one (5a, 5b, 5c, 5d, 5f, 5g, 5h, 7, 9, 11). A mixture of **1** or **6** or **8** or **10** HCl salt (5.0 mmol) and K_2CO_3 (1.38 g, 10.0 mmol) in acetonitrile (20 mL) was stirred at room temperature for 30 min. Then alkyl bromide **4a**−**g** (5.0 mmol) was added, dissolved in acetonitrile (5 mL). The mixture was stirred and refluxed until the consumption of the starting materials was complete (∼3 h). After the mixture was cooled, the inorganic salts were filtered off on sintered glass filter and washed with $CHCl_3$ (10 mL). The filtrate was evaporated, and the residue was partitioned between $CHCl_3$ (20 mL) and water (10 mL). The organic phase was separated, and the aqueous phase was washed with $CHCl_3$ (20 mL). The combined organic phase was dried ($MgSO_4$), filtered, and evaporated. The residue was purified by flash column chromatography (hexane/EtOAc) to give the title compounds in 35−71% yield.

3,5-Bis(4-fluorobenzylidene)-1-[(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl]piperidin-4-one (5e). To a solution of nitroxide **5c** (2.31 g, 5.0 mmol) in AcOH (25 mL), iron powder (2.8 g, 50.0 mmol) was added. The mixture was stirred at 60 °C for 30 min. After cooling, the reaction mixture was diluted with water (40 mL) and filtered. The filtrate was basified with solid K_2CO_3 to pH 8 (intensive foaming). The aqueous phase was extracted with $CHCl_3$ containing 10% MeOH (2 × 30 mL), and the combined organic phase was dried ($MgSO_4$), filtered, and evaporated. The residue was purified by flash column chromatography ($CHCl_3$/MeOH) to give the title compound as a yellow solid: 1.25 g (56%), mp 158−160 °C, R_f = 0.31 ($CHCl_3$/MeOH, 9:1). MS (EI) *m/z* (%): 448 (M^+ <1), 433 (12), 324 (13), 133 (56), 124 (100). Anal. Calcd for $C_{28}H_{30}F_2N_2O$: C 74.98, H 6.54, N 5.87. Found: C 75.01, H 6.68, N 5.70. ^{1}H NMR (CD_3OD): 7.73 (s, 2H); 7.46 (q, J = 15.4 Hz, ArH, 4H); 7.17 (t, J = 8.7 Hz, ArH, 4H); 5.38 (s, CH, 1H); 3.82, (s, $N(CH_2)_2$, 4H); 3.18 (s, CH_2, 2H); 1.12 (s, CH_3, 6H); 0.99 (s, CH_3, 6H).

In Silico Docking Simulations. Previously published work by our group[1,22] has demonstrated that these compounds act upon the signal transducer and activator of transcription 3 (STAT3) pathway. This phenomenon was investigated in more detail through in silico molecular docking simulations using the freely available program AutoDock (version 4.2).[40,41] The target macromolecule used in these studies was

a nontransformed murine STAT3 dimer downloaded from the RCSB Protein Data Bank (PDB code 3CWG).[42] Energy-minimized 3D molecular topographies of the DAP compounds were obtained using the Dundee PRODRG2 server.[43] To identify the site on the STAT3 dimer with the highest binding affinity for the DAP compounds, blind docking was accomplished using 0.625 Å grid spacing with 128 points in each of the *X*, *Y*, and *Z* directions. This grid covered the majority of the previously defined 3CWG molecular structure, including the entire SH2, linker, and DNA-binding domains, with partial coverage of the coil−coil domain.[42] Specific docking at the preferential site identified by blind docking was accomplished using 0.375 Å grid spacing with 100 points in each of the *X*, *Y*, and *Z* directions. Dockings were automatically ranked by AutoDock according to the lowest calculated binding energies (kcal/mol).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Synthesis and characterization of all new compounds and details of biological assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

A2780, human epithelial cancer cell line; MCF-7, human breast cancer cell line; H9c2, undifferentiated neonatal rat cardiomyoblasts; DAP, 3,5-bis(arylidene)-4-piperidones; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STAT3, signal transducer and activator of transcription 3; PARP, poly ADP-ribose polymerase; JAK, tyrosine kinase; VEGF, vascular endothelial growth factor; DNA, deoxyribonucleic acid; EPR, electron paramagnetic resonance

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