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RESEARCH ARTICLE

Resveratrol-salicylate derivatives as selective DNMT3 inhibitors and anticancer agents

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Abstract

Resveratrol is a natural polyphenol with plethora of biological activities. Resveratrol has previously shown to decrease DNA-methyltransferase (DNMT) enzymes expression and to reactivate silenced tumor suppressor genes. Currently, it seems that no resveratrol analogs have been developed as DNMT inhibitors. Recently, we reported the synthesis of resveratrol-salicylate derivatives and by examining the chemical structure of these analogs, we proposed that these compounds could exhibit DNMT inhibition especially that they resembled NSC 14778, a compound we previously identified as a DNMT inhibitor by virtual screening. Indeed, using *in vitro* DNMT inhibition assay, some of the resveratrol-salicylate analogs we screened in this work that showed selective inhibition against DNMT3 enzymes which were greater than resveratrol. A molecular docking study revealed key binding interactions with DNMT3A and DNMT3B enzymes. In addition, the most active analog, **10** showed considerable cytotoxicity against three human cancer cells; HT-29, HepG2 and SK-BR-3, which was greater than resveratrol. Further studies are needed to understand the anticancer mechanisms of these derivatives.

Keywords

Aspirin, chemoprevention, DNMT, docking, resveratrol

History

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Introduction

Resveratrol (3,4′,5-*trans*-trihydroxystilbene; Figure 1) is a naturally occurring polyphenol with a wide variety of biological properties. Resveratrol has been regarded as a phytoalexin (plant antibiotic), and it is produced by several plant species. The biological effects of resveratrol have been extensively studied *in vitro* and *in vivo*[1–5]; some of the reported effects of resveratrol include its anti-inflammatory[6], anticancer[7], antioxidant[8], cardioprotective[9], modulation of the estrogen receptor[10] and chemopreventive activity[11]. In this regard, resveratrol possesses an attractive chemopreventive profile, because it inhibits the proliferation of cancer cells *in vitro* without exerting significant cytotoxicity to normal cells[12]; it induces cancer cell apoptosis in several cell lines from different tissue types[13–15], and it significantly decreases tumor size *in vivo* using different cancer cells in xenograft models of rodents[16,17]. The mechanisms of action associated with the chemopreventive profile of resveratrol are varied and rather complex. In accordance with the current paradigm involving the design of ''multi-target'' drugs, and the relatively new concept known as polypharmacology[18], there is evidence supporting the multi-target profile of resveratrol. In this regard, resveratrol downregulates the expression or inhibits the activity of key enzymes and transcription factors involved in carcinogenesis, including (but not limited to) cyclooxygenase (COX) enzymes, inducible nitric oxide synthase, lipoxygenase, PI3-kinases, NF-κB, PPARγ, Sirt1, DNA-methyltransferases (DNMTs) and others[19].

DNMTs are a group of enzymes expressed by mammals in three active isoforms, namely the DNMT1, DNMT3A and DNMT3B (and one more regulatory enzyme identified as DNMT3L)[20]. Under normal physiological conditions, DNMTs are crucial for DNA methylation at cytosine residues[20]; specifically, DNMT3 functions as a initial (*de novo*) methylator, while DNMT1 is responsible for ''maintenance'' of the methylation during cell division[20]. However, aberrant methylation patterns (referred as ''epigenetic'') affecting certain genes and/or overexpression of DNMTs have been associated with many cancer types including lung, colorectal, prostate, breast, endometrial, gastric, hepatocellular, cervical and pancreatic cancers[21,22].

Experimentally, the selective inhibition of different DNMT enzymes has provided important clues to determine their role in physiology and pathophysiology. For example, it has been

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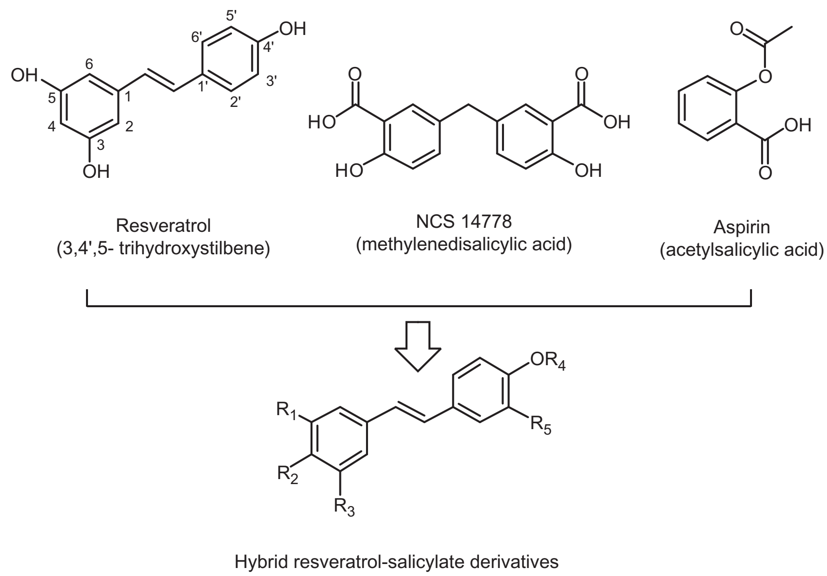


	R_1	R_2	R_3	R_4	R_5
Resveratrol	OH	H	OH	H	H
TMS	OCH_3	H	OCH_3	CH_3	H
3	H	OCH_3	H	CH_3	$COOCH_3$
4	H	OCH_3	H	Ac	$COOCH_3$
5	OCH_3	H	OCH_3	Ac	OCH_3
6	OCH_3	H	OCH_3	Ac	$COOCH_3$
7	OCH_3	H	OCH_3	CH_3	$COOCH_3$
8	H	OH	H	H	COOH
9	OH	H	OH	H	COOH
10	OH	H	OH	H	$COOCH_3$
11	H	OAc	H	Ac	COOH
12	OAc	H	OAc	Ac	COOH

Figure 1. Chemical structures of resveratrol, NSC 14778 and aspirin. The hybrid resveratrol-salicylate derivatives possess the combined chemical features of these three different types of agents; the methylene bridge in NCS 14778 is replaced by an ethylene linkage between a phenol on one side, and the salicylate on the other.

observed that DNMT inhibition reactivates ''silenced'' or hypermethylated genes, particularly tumor suppressor genes (genes associated with the expression of proteins that prevent tumor formation)[23,24]. Another important observation is that the concomitant incubation of DNMT inhibitors with chemotherapeutic agents[25,26], as well as radiotherapy[27], has shown significant synergistic effects of both of these therapeutic strategies. Finally, the inhibition of DNMT1 and DNMT3B has been shown to abrogate hepatitis C infection in hepatocellular carcinoma cells[28]. Consequently, it has been proposed that targeting the aberrant enzymatic activity of DNMTs could restore otherwise hypermethylated tumor suppressor genes, which is considered a promising strategy to prevent cancer initiation and cancer development[29,30].

The chemical structure and structural features required for a compound to display DNMT inhibition are described in the literature. According to the chemical structure, DNMT inhibitors can be classified in two main groups, namely the nucleos(t)ide and the non-nucleos(t)ide DNMT inhibitors[31–33]. Azacitidine (Vidaza®, Celgene) and Decitabine (Dacogen®, Astex) are two US FDA clinically-approved nucleoside DNMT inhibitors[33], whereas the compound MG98 represents an oligonucleotide. Representative examples of the non-nucleos(t)ide class of DNMT inhibitors are tryptophan derivative (RG108), quinoline derivatives (SGI-1027), alkyne derivatives, cyclopenta- and cyclohexathiophene derivatives, procainamide derivatives, genistein (natural flavonoid), curcumin, Psammaplin A (a marine natural compound) and hydralazine (see Figure 2 for chemical structures)[33].

Based on the observation that DNA methylation can be reversed by specific DNA repair mechanisms, the inhibition of hypermethylation of tumor suppressor genes is a promising strategy to prevent cancer initiation and development. This inhibition may take place over a long-period of time after administering either synthetic[34] or naturally occuring chemopreventive drugs[35]. Computational approaches have demonstrated the ability to identify DNMT inhibitors or compounds with demethylating properties that have novel scaffolds[32,36]. In this regard, a recent work published by Kuck et al.[37] reported the docking-based, virtual screening, and *in vitro* evaluation of more than 26 000 compounds from the National Cancer Institute (NCI) database on DNMT enzymes. In that paper, authors reported a series of small molecules with relatively high biochemical selectivity towards individual human DNMT enzymes. Using a multi-step docking approach of lead-like compounds with a homology model of the catalytic site of DNMT1, followed by experimental testing, authors identified seven new molecules with detectable DNMT1 inhibitory activity. The molecules identified in this study had diverse scaffolds, some of them not previously reported as DNMT inhibitors, such as a series of

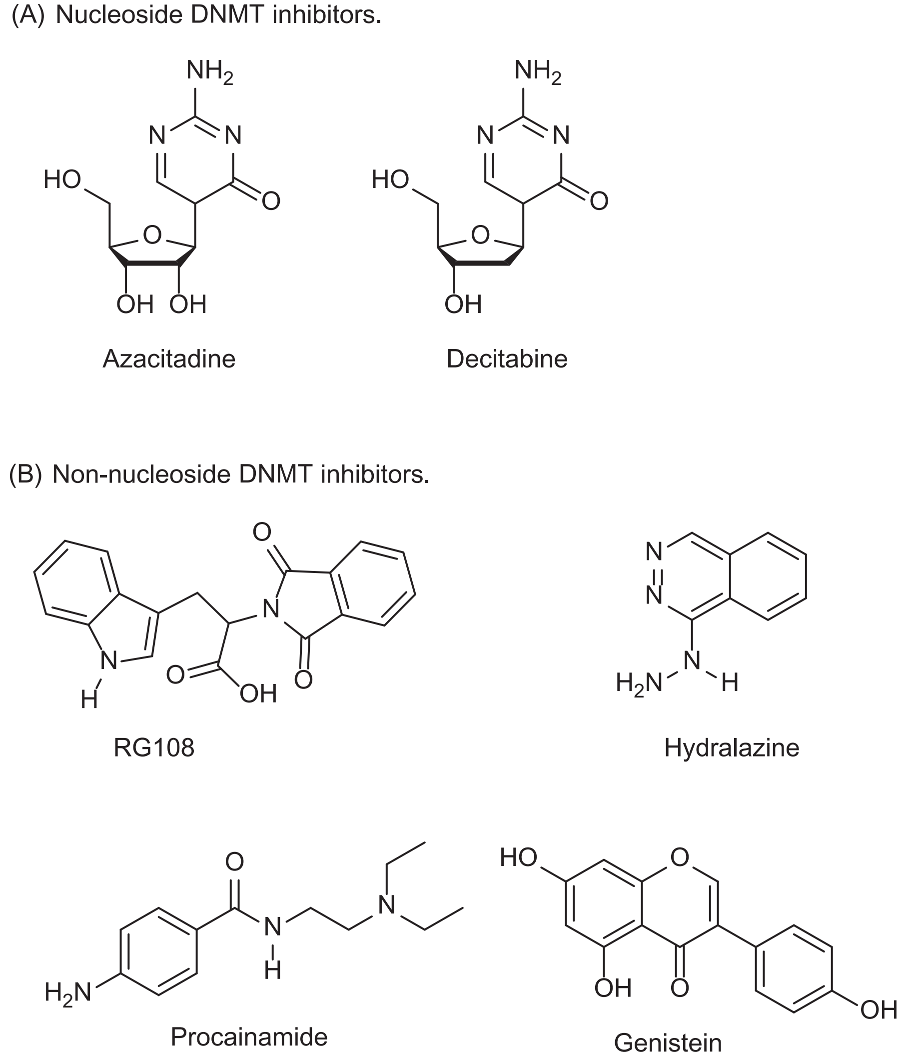


Figure 2. Chemical structures of representative examples of nucleoside and non-nucleoside DNMT inhibitors.

methylenedisalicylic acids, among which, the compound NSC 14778 (Figure 1) was one of the most potent compounds tested on DNMT1 and DNMT3B enzymes[37].

By analyzing the chemical structure of the scaffold present in methylenedisalicylic acids, and compare it to that of our recently reported resveratrol-salicylate analogs, in which we added a carboxylic acid group to one of the aromatic rings present in the polyphenol[38], we hypothesized that, in addition to the CYP1A1 inhibitory activity reported previously, these hybrid drugs could also inhibit the enzymatic activity of DNMT (Figure 1).

To the best of our knowledge, there are no reports in the literature describing that the direct inhibitory effect of resveratrol on DNMT enzymes, and the only report we could find on this regard, was published by Qin et al., who reported the effects of resveratrol on the expression of DNMT enzymes[39]. As part of an ongoing research work aimed at developing new cancer chemopreventive agents, we now report *in vitro* biological evaluation and the molecular modeling (docking) studies of a new series of resveratrol-salicylate derivatives with DNMT inhibitory activity. Our hypothesis was based on the idea that the addition of a carboxylic acid or its methyl ester, attached *ortho* to one of the phenol groups present in hydroxystilbenes, might confer resveratrol with a novel DNMT inhibitory profile, similar to that exerted by methylenedisalicylic acids described above. In this report, we identified compound **10** as the most active analog which showed greater than fourfold potency compared to resveratrol in inhibiting the DNMT3A enzyme. In addition, compound **10** exerted cell proliferation inhibition on three different human cancer cell lines (HT-29, HepG2 and SK-BR-3), suggesting that this chemical compound was more effective than the parent resveratrol under the same experimental conditions.

Materials and methods

Chemistry

We carried out the synthesis of hybrid resveratrol-salicylate derivatives **3–12** as described in our previous paper[38].

Inhibition of DNMT enzymes

The catalytic domains of DNMT3A/3B and full length DNMT 3L were purified as described previously by Hemeon et al.[40]. Full length DNMT1 was purified as previously described[41]. The dose–response experiments were performed against DNMT1 and DNMT3A/3B using the radiometric assay described by Hemeon et al.[40]. Briefly, the assay was conducted in the buffer containing 50 mM HEPES, 50 mM KCl, 5% glycerol and 1 mM DTT, pH 8.0. The inhibitors were preincubated in the buffer containing 1 μM of the corresponding enzyme or enzyme complex for 30 min, and the reaction was initiated by the addition of the substrate mix (1 μg dIdC substrate and 1.83 μM ^{3}H-*S*-adenosyl-L-methionine). The methylation reactions were allowed to proceed at ambient room temperature of 22 °C for 4 h (DNMT3B/3L) and overnight (DNMT3A/3L). Subsequently, 6 μL of the reaction was spotted on a 1.2 cm × 1.2 cm DE81 Anion Exchanger exchange filter paper squares. Each reaction was spotted three times. The filter paper was allowed to dry for 15 min, and washed twice with 0.2 M ammonium bicarbonate, followed by deionized double-distilled water and ethanol. The filter paper was then put in scintillation vials, followed by the addition of 0.5 mL of deionized double distilled water and then 5 mL of scintillation fluid. The signal was monitored using a Liquid Scintillation Analyzer (Perkin Elmer Tri-Carb 2910 TR, Waltham, MA) and percent inhibition was calculated as previously described[42].

Molecular modeling

Proteins. The crystal structures of human DNMT1 (PDB ID: 3SWR) and DNMT3A (PDB ID: 2QRV) were retrieved from the Protein Data Bank (PDB), whereas for the DNMT3B structure we used the homology model, we previously published for this isozyme[37]. The structures were prepared and submitted to a geometry optimization protocol (OPLS force field) by using the Protein Preparation Wizard protocol of Schrödinger software (New York, NY) using the default settings[43].

Ligands. Compounds **3–12**, 3,4′,5-*trans*-trimethoxystilbene (**TMS**) as well as resveratrol were built and submitted to a geometry optimization protocol employing the AMBER99SB force field in UCSF Chimera 1.9[44].

Docking. Molecular docking studies were performed using AutoDock 4.2 software[45] (San Diego, La Jolla, CA). In these studies, we evaluated the compounds in the DNMT catalytic site in the presence and absence of the co-factor. We used a grid box of 80 × 80 × 80 points with a grid spacing of 0.375 Å that covers the catalytic pocket and the co-factor binding site. The Lamarckian genetic algorithm was used as a search method. A total of 20 runs were carried out with a maximal number of 5 000 000 energy evaluations and initial population of 150 conformers. The best binding modes for each molecule were selected for the analysis. We have previously used AutoDock to model DNMT inhibitors[37].

Cytotoxicity

Human colorectal adenocarcinoma HT-29 (ATCC HTB-38, Manassas, VA), human hepatoma HepG2 cells (ATCC HB-8065, Manassas, VA) and human mammary gland/breast SK-BR-3 cells (ATCC HTB-30, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were grown in 75-cm^2 tissue culture flasks at 37 °C in a 5% CO_2 humidified incubator. To evaluate the antiproliferative effect of the most active compound **10**, resveratrol and its natural analog **TMS**, we carried out a series of MTT assays using a published procedure[46] with minor modifications. All test compounds were dissolved in DMSO and tested at a final concentration range of 0.03–125 μM, over a 24-h incubation period. The final concentration of DMSO in culture media was fixed at 0.5% (v/v). The corresponding IC_{50} values were calculated from the cell growth inhibition curve using GraphPad Prism software (GraphPad software Inc., La Jolla, CA) (IC_{50} values represent an average of three different experiments, in triplicate).

Results and discussion

During the last decade, the evolution of epigenetics and the validated association of this biological process with many disorders such as cancer[47], Alzheimer[48], cardiovascular diseases[49] and diabetes[50] have been the subject of scientific research. These epigenetic mechanisms are regulated by multiple proteins including DNMT enzymes[51]. DNMTs catalyze the transfer of a methyl group from the substrate *S*-adenosylmethionine (SAM), to DNA cytosine residues (called ''CG sites'')[20]. DNMT1 specifically methylates hemi-methylated DNA, while DNMT3A and DNMT3B bind to unmethylated DNA to carry out *de novo* methylation[20]. It has been proposed that small molecule inhibitors of DNMT enzymes can bind either at the catalytic binding pocket binding site of DNA or at the binding site of the cofactor *S*-adenosylhomocysteine (SAH)[52], or both, depending on the structure of the inhibitor. The latter is particularly applicable if the structure of the inhibitor has a ''long'' scaffold such as SGI-1027[53].

Compound NSC14778 (Figure 1), a methylenedisalicylic acid was reported by Kuck et al. after implementing a virtual screening protocol on more than 26 000 compounds from a NCI database, provided a useful lead scaffold to design new molecules with potential DNMT inhibitory activity[37]. In this regard, we have recently reported the chemical synthesis and CYP1A1 inhibitory profile of a new series of hybrid resveratrol-salicylate analogs with promising chemopreventive activity[38]; after we re-examined the chemical structures of these derivatives, we recognized a potentially useful pattern: by replacing the central methylene group in NSC14778 for the ethylene (CH=CH) moiety present in stilbenes. It is noteworthy that we identified some structural similarities between NSC14778, and our recently reported salicylate-resveratrol analogs, so that it was reasonable to predict certain degree of DNMT inhibition by our molecules (however, please note that we did not start the design of our salicylate-resveratrol derivatives based on the docking pose of NSC14778). We hypothesized that these compounds could exert significant inhibition of DNMT enzymes, for two reasons.

First, the new salicylate moiety on resveratrol (Figure 1) would resemble the salicylate group in NSC14778, which has been reported as ''essential'' for DNMT inhibition[37]; second, literature reports have shown that resveratrol is capable of reducing the expression of DNMT enzymes, reactivating previously hypermethylated tumor-suppressor genes[23,24,34].

In vitro DNMT inhibition

To study the *in vitro* DNMT inhibition exerted by the salicylate-resveratrol analogs reported previously[38], we used a filter paper based Scintillation Proximity Assay (SPA)[42]. We used the well-known DNMT inhibitor (although structurally unrelated) *S*-adenosyl-L-homocysteine (SAH)[54,55] as the standard which showed $IC_{50} = 2$ μM on DNMT1. For comparison purposes, we also used the parent stilbene resveratrol, which showed inhibition on DNMT3B ($IC_{50} = 65$ μM) and DNMT3A ($IC_{50} = 105$ μM), but no activity on DNMT1 (IC_{50} higher than 300 μM, Table 1). These results suggest that the parent polyphenol shows selective inhibition of the DNMT3B isozyme. This observation is somewhat related to a recent study reported by Qin et al.[39] in which his group reported a significant reduction in the expression of DNMT3B after a 21-week treatment period to rats with resveratrol. Interestingly, this treatment did not significantly reduce the expression of the DNMT1 enzyme[39]. Nevertheless, it is important to distinguish that our study measured enzyme activity, whereas that of Qin et al. determined protein expression.

Another compound that could be a potential DNMT inhibitor is the methylated version of resveratrol, or 3,4′,5-trans-trimethoxystilbene (**TMS**), which has previously displayed an enhanced anticancer profile compared to resveratrol[46]. In this regard, we observed no significant inhibition of DNMT enzymes at the

Table 1. Concentration (μM) of test compounds required to inhibit by 50% the enzymatic activity of DNMT1, DNMT3A, and DNMT3B.

	IC_{50} (μM)		
Compounds	DNMT3A/3L	DNMT3B/3L	DNMT1
3	282	>300	NI[a]
4	>300	>300	NI[a]
5	>300	>300	NI[a]
6	>300	>300	NI[a]
7	>300	>300	NI[a]
8	281	156	NI[a]
9	40	52	NI[a]
10	25	62	NI[a]
11	186.6	190	NI[a]
12	100	215	NI[a]
TMS	>300	>300	NI[a]
Resveratrol	105	65	>300
SAH	ND[b]	0.25[c]	2

Results are expressed as IC_{50} values using a cell-free biochemical assay. To generate the enzyme inhibition curves, duplicate reactions were performed for each concentration; IC_{50} values were calculated using the GraphPad Prism v6 software.
[a]NI, no inhibition at the maximum test compound concentration (300 μM).
[b]ND, not determined.
[c]Taken from Ref.[37].

highest test compound concentration (300 μM). This suggests that the free hydroxyl groups present in resveratrol are essential to exert inhibition of the DNMT enzymatic activity. As far as we are concerned, our study is the first one reporting the apparent lack of activity of **TMS** on DNMT1, DNMT3A and DNMT3B enzymes (Table 1). A similar effect (i.e. loss of enzymatic inhibitory activity with DNMT1 upon methylation of a hydroxyl group) was noted for a sulfonamide DNMT inhibitor recently identified by high-throughput screening[56,57].

Once we analyzed the inhibitory profile of compounds described above, we started the screening evaluation of the new hybrid salicylate-resveratrol derivatives. According to our results, derivatives possessing methoxy groups (**3–7**) at any position on the stilbene structure were practically inactive at the highest test compound concentration (300 μM), which is in accordance with the results obtained for **TMS**. However, we also observed that derivatives possessing free hydroxyl groups (**8–10**) were significantly more potent than their methoxylated counterparts, but their inhibitory profile was significant only on DNMT3A and DNMT3B enzymes, not on DNMT1. In this regard, the most active compounds in the series were compounds **9** and **10**, which showed significant inhibition on both DNMT3A (IC_{50} = 40 and 25 μM respectively) and DNMT3B (IC_{50} values = 52 and 62 μM, respectively).

Compounds possessing an acetyl group (mimicking an acetylsalicylic acid moiety), either on a 4′- or a 3,5-pattern, are not as potent as those having the free hydroxyl groups. Interestingly, the negative effect of adding acetyl groups on DNMT3 inhibition is milder than that of adding methoxy groups, given that compounds **11** and **12** still showed some degree of inhibition on both DNMT3A and DNMT3B enzymes (IC_{50}'s in the 100–215 μM range; Table 1). The observation that methoxy groups reduce DNMT inhibition seems to be in agreement with a recent report by Rilova et al., in which they reported that dimethoxytriazine groups decreased the DNMT3A inhibitory activity of quinolone-based DNMT inhibitors[58].

A detailed comparison between molecules **8** and **9**, both possessing free hydroxyl groups, suggests that two phenol groups at positions 3 and 5 (compound **9** IC_{50} values = 40 μM –on DNMT3A–, and 52 μM –on DNMT3B–) exert a better enzyme inhibitory profile than having only one at the 4-position (compound **8** IC_{50} values = 281 μM –on DNMT3A–, and 156 μM –on DNMT3B–).

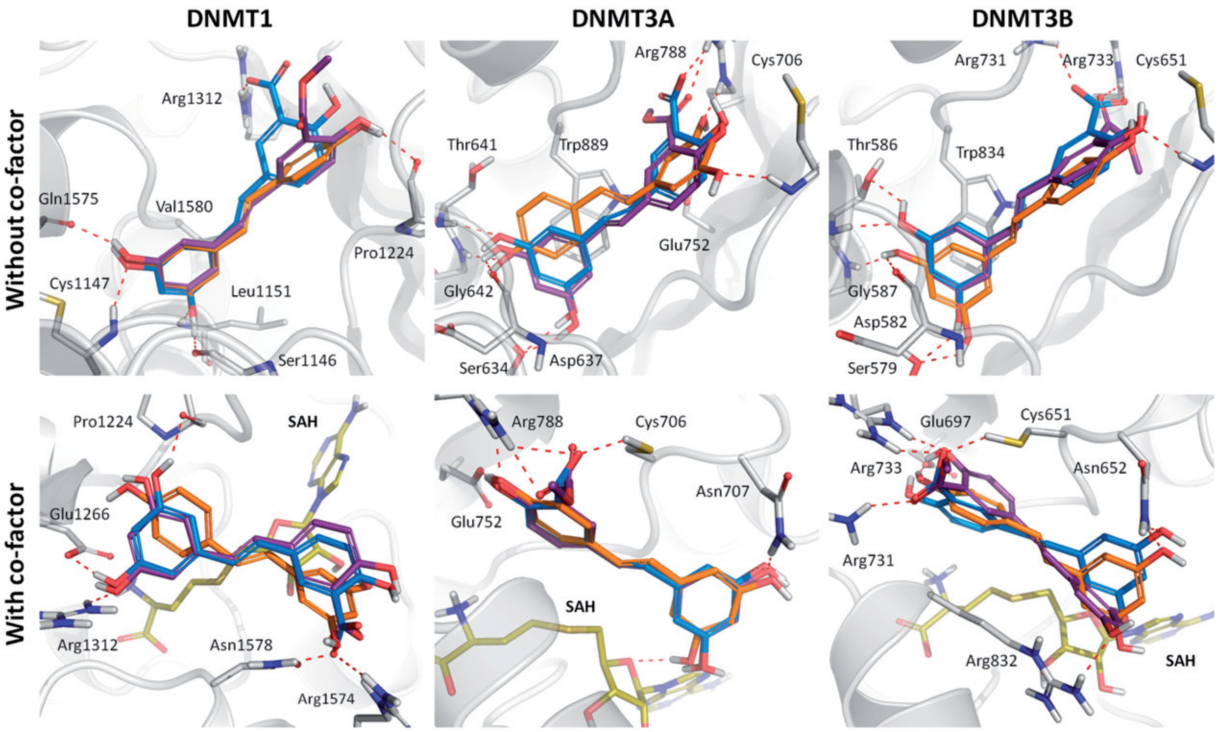
To complement the structural analysis of compounds **3–12**, we studied the effects of the carboxylic acid group on the stilbene scaffold, which according to previous reports, seems to be an essential requirement for DNMT1 inhibition. This requirement has been previously described in different molecules. Analog series of drugs having a carboxyl group are in general more potent DNMT1 inhibitors than those not having it[34,59]. This observation has been studied using molecular modeling (docking) studies, and it has been predicted that carboxylate anions are able to form hydrogen bonds with key amino acid residues in the active site of DNMT1[34,59]. Nevertheless, according to our results and the experimental conditions we used, for DNMT3A and DNMT3B, the presence of the carboxylic acid group on the stilbene scaffold seems to be significant only when the phenol groups are free. As it can be observed with our small library of hybrid salicylate-resveratrol derivatives, compounds possessing a free carboxylic acid (**8**, **9**, **11** and **12**), a carboxylate methyl ester (**3**, **4**, **6**, **7** and **10**) or no carboxylic acid at all (compound **5**) did not show any inhibition on DNMT1, even at concentrations as high as 1 mM (results not shown). In this regard, a recent study by Asgatay et al. showed that a *N*-phthaloyl-L-tryptophan derivative, in which a carboxylate group was replaced by an amide function, can still display some activity toward DNMT1. Therefore, authors proposed that the essential role of the carboxyl group is still ''inconclusive''[60].

As far as DNMT3A/3B inhibition is concerned, it is still not clear if the presence of a carboxyl group is required for a drug to exert binding interactions in the active site of DNMT3 enzymes. Nevertheless, recent developments with small molecule inhibitors have shown that *in vitro* DNMT3A inhibition is possible without the presence of carboxylate groups[58]. Thus, results of this work showed that compounds bearing either a free carboxylic acid (**9**), or a carboxylate methyl ester (**10**), exerted a better inhibitory profile than resveratrol against both DNMT3 enzymes (Table 1).

Molecular modeling

To test *in silico* the ability of the test compounds to interact with the catalytic site of DNMT enzymes, we carried out molecular modeling (docking) simulations, in which we assessed the ability of hybrid salicylate-resveratrol derivatives to exert binding interactions with key amino acid residues in the enzyme's active site. We did these experiments in the presence and in the absence of the co-factor SAH according to a previously reported protocol[52].

Figure 3 shows the binding mode for the parent compound (resveratrol), and the active compounds **9** and **10** within the human DNMT enzyme binding sites, in the presence and in the absence of the co-factor. The table below Figure 3 summarizes the calculated binding free energies for each binding mode. The binding free energies as calculated by Autodock, and the binding modes of the remaining compounds are reported in Table 2 and Figure 4, respectively. According to our molecular modeling results, the docking scores calculated for resveratrol, compound **9** and compound **10** in the active sites of both DNMT3A and DNMT3B (in the presence and absence of the co-factor) are, overall, more favorable (more negative), than those values obtained with DNMT1. Despite the well-known number of approximations considered in calculating docking scores[61], this is in *good qualitative agreement* with the trend observed experimentally. In the docking study performed in the absence of the co-factor, the presence of a π–π interaction with Trp889 and



Compound	DNMT1		DNMT3A		DNMT3B	
	Without co-factor	With cofactor	Without co-factor	With cofactor	Without co-factor	With cofactor
9	−8.13	−7.67	−9.66	−8.17	−8.85	−7.54
10	−7.43	−6.42	−9.27	−7.01	−8.99	−8.82
Resveratrol	−7.74	−5.83	−9.03	−6.57	−7.95	−6.22

Figure 3. Comparison of the binding modes calculated for compounds **9** (blue), **10** (purple), and resveratrol (orange) as predicted by AutoDock 4.2, in the presence and in the absence of the co-factor SAH (yellow) in the active site of DNMT1, DNMT3A and DNMT3B enzymes.

Table 2. Calculated binding free energies of resveratrol-salicylate analogs in human DNMTs.

	DNMT1		DNMT3A		DNMT3B	
Compound	Without cofactor	With cofactor	Without cofactor	With cofactor	Without cofactor	With cofactor
3	−7.52	−6.58	−8.11	−7.08	−7.32	−7.16
4	−8.47	−5.83	−8.99	−5.84	−8.02	−6.78
5	−8.26	−6.26	−8.52	−6.27	−8.76	−6.17
6	−7.05	−6.39	−5.06	−6.29	−8.28	−7.02
7	−7.44	−6.14	−8.40	−5.76	−7.82	−7.85
8	−8.37	−6.91	−9.41	−7.84	−9.05	−8.54
9	−8.13	−7.67	−9.66	−8.17	−8.85	−7.54
10	−7.43	−6.42	−9.27	−7.01	−8.99	−8.82
11	−−8.94	−7.21	−7.65	−7.72	−9.33	−8.23
12	−7.95	−8.96	−8.42	−7.50	−7.88	−8.44
TMS	−7.36	−6.02	−7.95	−5.67	−8.23	−7.53
Resveratrol	−7.74	−5.83	−9.03	−6.57	−7.95	−6.22

Trp834 was observed in the DNMT3A and DNMT3B structures, respectively. It is noteworthy that the tryptophan is absent in the structure of the DNMT1, which may explain the differences in binding energies and the lack of activity on the DNMT1 isoform.

In the study carried out in the presence of the co-factor, we observed interactions of the ligands with the catalytic cysteine, glutamic acid and arginine in both DNMT3A (Cys706, Glu752 and Arg788) and DNMT3B (Cys651, Glu697 and Arg733) active sites, which in previous studies have proven to be a primary interaction for enzyme inhibition. In this regard, Cys651 has previously shown to be a key site for binding interactions between the antibiotic Nanaomycin and the DNMT3B enzyme[62]. Consequently, these docking results allowed us to hypothesize that regardless of the operating inhibition mechanism (with or without the co-factor), these binding interactions may offer a plausible explanation for the observed selectivity toward DNMT3

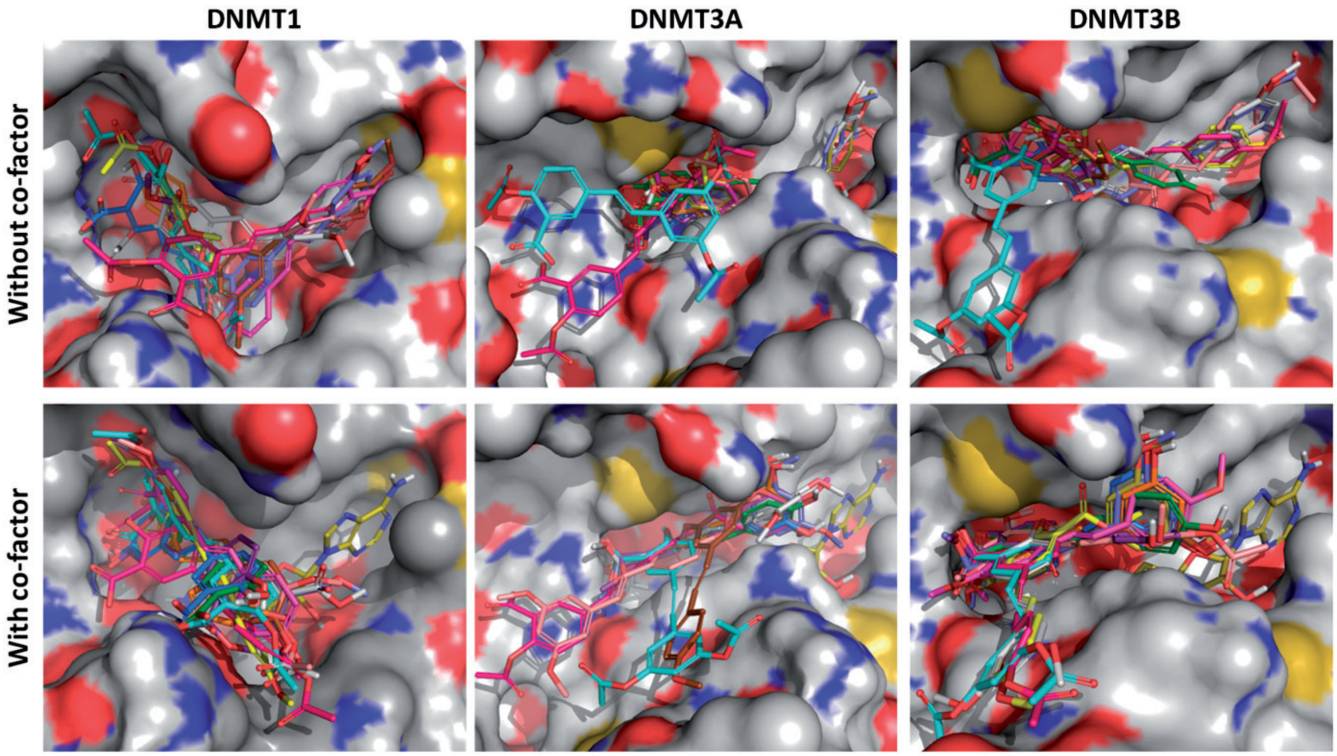


Figure 4. Comparison of the binding modes of compounds **3** (magenta), **4** (yellow), **5** (pink), **6** (gray), **7** (violet), **8** (green), **9** (blue), **10** (purple), **11** (pink), **12** (cyan), **TMS** (brown) and resveratrol (orange) predicted by AutoDock 4.2 in the presence and absence of co-factor SAH of DNMT1, DNMT3A and DNMT3B.

enzymes by the test compounds, including the parent resveratrol. It is noteworthy that the presence of the 3,5-dihydroxyphenyl group (also called resorcinol) is important for the interaction of the ligands in both studies, suggesting that the test compounds should have this group for the inhibition of DNMT3 isoforms.

In a previous study[37], Kuck et al. reported multiple hydrogen bonds between NSC14778 and key amino acid residues in the active site of DNMT1, particularly Arg174 and Cys88. These residues were shown to play an essential role in the mechanism by which DNMT1 carries out methylation of DNA methylation. In this regard, NSC14778 was able to block the active site of DNMT1 by exerting non-covalent interactions[37]. Comparatively speaking, the observations that our derivatives showed weak binding energies in the active site of the DNMT1 (Table 2), and were inactive *in vitro* on this enzyme (Table 1), suggests that the mechanism of action of the resveratrol-salicylate derivatives is different than that of methylenedisalicylic acid NSC14778.

Cytotoxicity in culture cells

The promising inhibitory profile observed for compound **10**, along with the corresponding molecular modeling (docking) studies, and the observation that epigenetic modifications in cancer cells are essential for cell proliferation, we evaluated the effects of compound **10** on *in vitro* cell proliferation. To carry out this, we used three different human cancer cell lines, namely HT-29 cells (colorectal), HepG2 cells (liver) and SK-BR-3 cells (breast). In these cell lines, DNMT-mediated epigenetic regulations have been recently confirmed[25,63,64]; the results are summarized in Table 3. Interestingly, compound **10** exerted a stronger cell proliferation inhibition than that exerted by the parent resveratrol in all three cancer cells, and it was more active than **TMS** on HepG2 and SK-BR-3 cells. In this regard, it has been reported that **TMS**, being a more lipophilic stilbene than resveratrol (and consequently, more likely to cross cell membranes), demonstrated a higher cell proliferation inhibition than resveratrol in cancer cells[46]. The ability of **TMS** (as well as compound **10**) to inhibit DNMT3 activity does not exclude other mechanisms by which this hybrid molecule could decrease cell proliferation. In fact, there is considerable evidence backing up the multi-target profile exerted by resveratrol, which may be applicable to its salicylate hybrid **10**; nevertheless, further studies are needed to investigate other mechanisms of anti-proliferative activity exerted by this compound.

Table 3. Concentration (μM) of the test compounds required to inhibit cell proliferation by 50 % (IC_{50}) using the MTT assay.

	IC_{50} (μM)		
Compounds	HT-29	HepG2	SK-BR-3
10	44.3	18.9	11.3
Resveratrol	130.0	54.9	110.3
TMS	14.0	>100	111.8

Each IC_{50} value represents the mean of three different experiments in triplicate. To generate the cell proliferation inhibition curves, six concentrations (in the 0.03–125 μM range) were used for each compound. IC_{50} values were generated using the GraphPad v6 Prism software.

In a previous study[38], we reported the CYP1A1 inhibitory profile of compounds **3–12**, in which we elaborated on the chemical features required for hybrid molecules to exert inhibitory activity on CYP1A1. Compound **10** was not as effective as other molecules inhibiting the CYP isoform; however, the binding interactions make this molecule an effective DNMT3 inhibitor, despite its lack of activity on CYP enzymes. These observations suggest that the overall design of hybrid salicylate-resveratrol analogs is flexible enough to offer preferential inhibition against at least these two proteins (CYP1A1 and DNMT3).

Conclusion

We showed that the hybrid salicylate-resveratrol scaffold is a promising alternative to the parent stilbene resveratrol and its methylated analog **TMS** as a DNMT inhibitor. Derivatives **9** and **10** showed a significant and selective inhibitory profile on DNMT3A and DNMT3B enzymes, which are 2–4 times more potent than that exerted by resveratrol under the same experimental conditions. Structure–activity relationships showed that free hydroxyl groups are required to exert DNMT3 inhibition, and this pattern is better in analogs having phenols in positions 3 and 5 of a stilbene. The presence of the salicylate group in the resveratrol's structure produced an enhanced inhibitory profile only when there are free phenol groups on the stilbene. Compound **10** showed an improved *in vitro* cell proliferation inhibition compared to resveratrol and **TMS** on at least two human cancer cells, suggesting that compound **10**, and possibly compound **9**, are promising candidates worth evaluating *in vivo*, to further understand their potential anticancer/chemopreventive properties.

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Declaration of interest

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