



## DNA site-specific N3-adenine methylation targeted to estrogen receptor-positive cells

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### ABSTRACT

A compound that can target cells expressing the estrogen receptor (ER), and produce predominantly 3-MeA adducts in those cells has been designed and synthesized. This compound produces mainly the 3-MeA adduct upon reaction with calf thymus DNA, and binds to the ER with a relative binding affinity of 51% (estradiol = 100%). The compound is toxic to ER-expressing MCF-7 breast cancer cells, and pre-treatment with the ER antagonist fulvestrant abrogates the toxicity. Pre-treatment of MCF-7 cells with netropsin, which inhibits N3-adenine methylation by the compound, resulted in a threefold decrease in the toxicity. These results demonstrate the feasibility of this strategy for producing 3-MeA adducts in targeted cells.

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### 1. Introduction

DNA alkylation by small molecules is a strategy that has been commonly used to destroy malignant cells, and DNA-alkylating drugs play an important role in cancer chemotherapy.<sup>1,2</sup> However, most of these drugs have little specificity, either for cancer cells, or for particular DNA sequences, and their selective destruction of cancer cells depends upon the rapid proliferation of cancer cells compared to normal cells. The potency of these drugs is usually severely diminished due to their widespread uptake by normal cells, and due to non-specific DNA alkylation, leading to the formation of multiple kinds of DNA lesions, not all of which result in cytotoxic consequences. In addition, the inability to target these drugs to specific cells results in unwanted side-effects (e.g., immune suppression, hair loss, gastric irritation, etc.). More significantly,

the indiscriminate DNA damage caused by these compounds can sometimes lead to mutations in normal cells which are believed to be responsible for cases of secondary cancer, typically leukemia, following chemotherapy with DNA-alkylating drugs.<sup>3–6</sup>

Several strategies have been developed in recent years in order to overcome the therapeutic shortcomings of DNA-alkylating compounds. One strategy has been to confer cell-targeting ability to alkylating compounds, typically by using a ligand that binds to a tumor cell-specific enzyme or receptor in order to improve delivery of the alkylating agent to tumors.<sup>7–12</sup> Another strategy has been to increase the concentration of the alkylating agents in the vicinity of DNA (and thereby reducing the loss of the agent due to reaction with other cellular nucleophiles) by conjugating specific DNA-binding moieties to the alkylating agents.<sup>13–22</sup> Surprisingly, there appear to be no approaches that combine both cell-targeting and DNA-binding strategies.

We describe here a strategy that combines cell-targeting ability with DNA sequence specific alkylation for producing N3-methyladenine (3-MeA) DNA adducts in the targeted cells. The ability to form 3-MeA adducts in targeted cells affords several attractive possibilities. 3-MeA is a highly cytotoxic lesion<sup>23</sup> with low mutagenicity.<sup>24</sup> 3-MeA lesions are repaired via the Base Excision Repair pathway as shown in Figure 1.<sup>25</sup> High levels of 3-MeA adducts lead to over-activation of poly(ADP-ribose) polymerase (PARP) during

**Abbreviations:** MeA, N3-methyladenine; 7-MeG, N7-methylguanine; EC<sub>50</sub>, half maximal effective concentration; ER, estrogen receptor; ER+, estrogen receptor-positive; IC<sub>50</sub>, half maximal inhibitory concentration; Me-lex, methyl 3-((1-methyl-5-((1-methyl-5-(propylcarbamoyl)-1H-pyrrol-3-yl)carbamoyl)-1H-pyrrol-3-yl)amino)-3-oxopropane-1-sulfonate; MMS, methylmethane sulfonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PARP, poly(ADP-ribose); RBA, relative binding affinity; TR-FRET, time resolved fluorescence resonance energy transfer.

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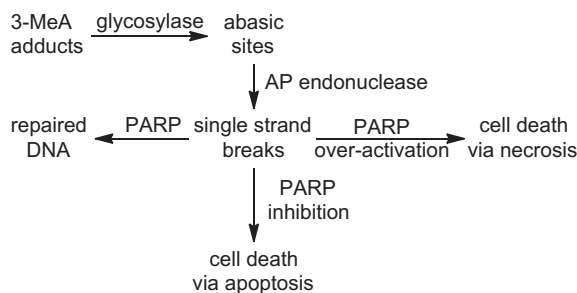


Figure 1. 3-MeA processing via Base Excision Repair.

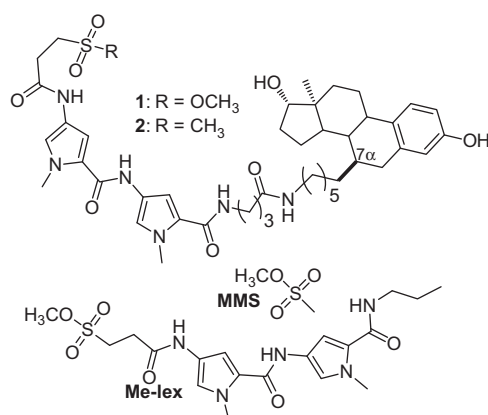


Figure 2. Compounds used in this study.

this repair process, which rapidly depletes cellular ATP and causes cell-death by necrosis.<sup>26,27</sup> However, if PARP is inhibited, the repair process results in an accumulation of DNA single strand breaks, and consequently, the cell dies by apoptosis.<sup>26</sup> Furthermore, 3-MeA adducts, under certain conditions, have been implicated in the triggering of an autoimmune response.<sup>28</sup> Transgenic mice, incapable of repairing 3-MeA adducts, when exposed to a pancreatic  $\beta$ -cell specific DNA-methylating agent, develop delayed autoimmune diabetes, whereas wild-type animals subjected to the same treatment do not.<sup>28</sup> Thus, the ability to produce these 3-MeA lesions in targeted cells, in combination with PARP inhibitors, could result in the selective destruction of the targeted cells, provide the ability to modulate the mechanism of cell-death, and could enable further investigation into the possible role of 3-MeA in eliciting autoimmune responses. Thus, in addition to providing attractive therapeutic alternatives, the ability to generate 3-MeA lesions in targeted cells would provide useful tools for research.

In this article, we present the design and synthesis of **1** (Fig. 2), and demonstrate the successful implementation of our strategy by presenting preliminary evidence for the ER-mediated 3-MeA formation by **1** in MCF-7 cells, and its consequences. The non-alkylating stable analog of **1**, compound **2** (Fig. 2), was also synthesized and used as a control in this study, and was also used to determine the ER-binding of the compound. The results demonstrate the feasibility of using this strategy for selectively targeting any particular cell-type that expresses a unique enzyme or receptor, and producing cytotoxic, non-mutagenic 3-MeA adducts in those cells.

## 2. Results and discussion

### 2.1. Design

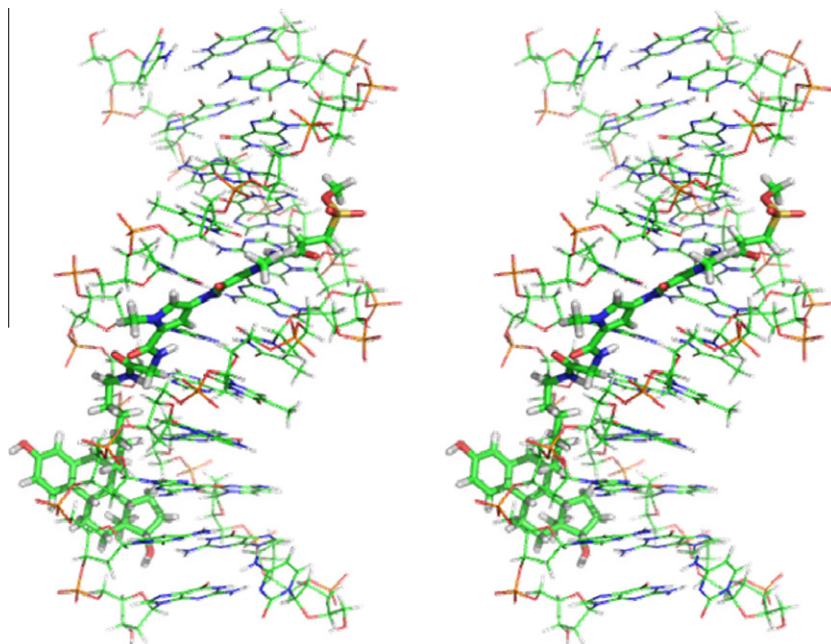
Compound **1** has been designed to target estrogen receptor-positive (ER+) cells and produce predominantly 3-MeA adducts in

those cells. The design of **1** was inspired by Me-lex (Fig. 2, (1-methyl-4-{1-methyl-4-[3-(methoxysulfonyl)propanamido]pyrrole-2-carboxamido}pyrrole-2-carboxamido)propane) which has been shown to produce, almost exclusively (over 95%), 3-MeA DNA adducts.<sup>22</sup> The 3-MeA lesions formed by Me-lex have been shown to be highly cytotoxic and have low mutagenicity.<sup>24</sup> Me-lex lacks any cell targeting ability, and most likely enters cells by passive diffusion. The potent cytotoxicity of Me-lex, and the absence of mutagenicity upon exposure to Me-lex, makes it an attractive candidate for targeted delivery to cells. In **1**, Me-lex is conjugated to estradiol in order to deliver it selectively to cells which express the estrogen receptor (ER). Estradiol was selected as the cell-targeting ligand due to its demonstrated ability to selectively deliver agents to ER+ cells,<sup>29–31</sup> and the therapeutic relevance of ER-targeting for breast cancer. Most breast cancer cells over express the ER,<sup>32,33</sup> and it has also been shown that using agents that have high affinity for the ER, one can achieve increased concentration ratios between target tissues that express the ER and non-target tissues.<sup>33</sup>

The size, composition, and point of attachment of the linker to the estradiol moiety and Me-lex were important considerations in the design of **1**. The 7 $\alpha$ -position (Fig. 2) of estradiol was chosen as the site of attachment because alkyl substituents at this position do not diminish estradiol's high binding affinity for the ER.<sup>29</sup> Structure-activity studies of a series of 7 $\alpha$ -substituted estradiols have also shown that the length of the side-chain at this position is crucial for ER-binding and function,<sup>34</sup> and tethers of at least six carbons in length are needed to achieve significant ER-binding.<sup>29</sup> Me-lex achieves its remarkable selectivity in DNA methylation due to the binding of its bis-pyrrole triamide unit within the minor groove of DNA at A/T rich regions using a combination of hydrogen bonding and van der Waal interactions. Molecular dynamic simulations with Me-lex<sup>35</sup> had indicated that the alkyl group at the N-terminus of this molecule did not participate in any key interactions within the groove, and would, therefore, be a suitable site for attachment of the estradiol ligand. Similar molecular dynamic simulations of the binding of **1** with the Dickerson dodecamer d(CGCGAATTCGCG)<sub>2</sub> (unpublished work) indicated that the bis-pyrrole moiety was able to maintain its interactions within the minor groove at the AATT core of the duplex oligomer even with the estradiol ligand attached. Furthermore, the estradiol ligand itself penetrated into the minor groove and established favorable interactions within the groove (Fig. 3).

### 2.2. Synthesis

Compounds **1** and **2** were synthesized using the sequence outlined in Scheme 1. The strategy adopted was to synthesize the bis-pyrrole component containing part of the linker unit functionalized with a terminal carboxylic acid (**7**) and condense it with the estradiol unit containing the rest of the linker functionalized with a terminal amine (**12**). Compound **3** (prepared by following published procedures<sup>36</sup>) was condensed with the appropriate amino ester to give **4a** or **4b**. Reduction of the nitro-group of **4a** or **4b** by high pressure hydrogenation, followed by condensation of the resultant aryl amine with another unit of **3** resulted in compounds **5a** or **5b**, which now contained the DNA-binding core of the molecules. For the synthesis of **1**, the nitro-bis-pyrrole ethyl ester **5a** was subjected to high pressure hydrogenation, and the resultant amine was reacted with acryloyl chloride to prepare **6**. Hydrolysis of the ethyl ester in **6** at room temperature resulted in carboxylic acid **7** which was now suitable for condensation with the appropriate ER-binding estradiol component. This estradiol compound, **12**, bearing an amino-hexyl substituent at the 7 $\alpha$ -position, was synthesized following published procedures.<sup>37,38</sup> Condensation of **12** with **7** resulted in the formation of **8**. The acidic conditions used



**Figure 3.** Molecular dynamics simulations: stereo view of **1** bound within minor groove of Dickerson dodecamer.

during the work-up procedure removed the TBDMS protecting group. Reaction of **8** with sodium bisulfite resulted in the Michael addition of sulfonate, and subsequent acid work-up gave **9**. Methylation of the sulfonic acid in **9** to obtain the final desired compound **1** was accomplished using 1-methyl-3-(*p*-tolyl)triaz-1-ene.

For the synthesis of the stable analog **2**, compound **5b** was first reduced under high pressure hydrogenation conditions, and the resultant amine was condensed with **13** (which was synthesized from 3-methylthiopropionic acid) to form **10**. The acid hydrolysis of **10** resulted in the formation of **11** in quantitative yields. Condensation of carboxylic acid **11** with **12**, and subsequent acidic work-up resulted in the desired compound **2**. Two other methylating agents, Me-lex (Fig. 2), which exhibits sequence specific DNA-binding but no cell targeting ability, and methylmethane sulfonate (MMS, Fig. 2), which represents the methylating terminus of **1** and has neither cell-targeting nor sequence-specific DNA-binding capability, were also required for this study. MMS was commercially obtained, and Me-lex was synthesized following published procedures.<sup>39</sup>

### 2.3. DNA methylation

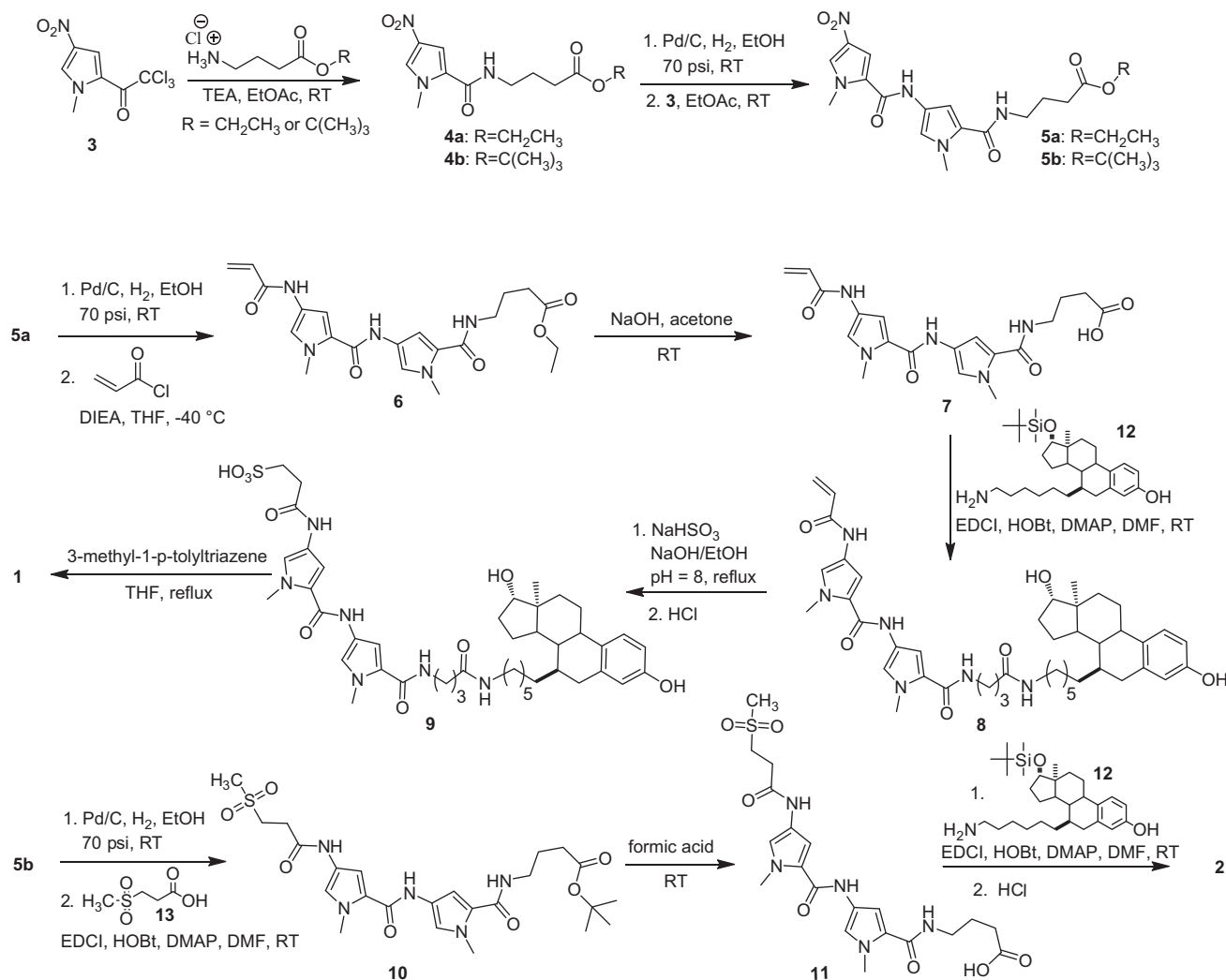
In order to determine the DNA-methylating profile of **1**, it was reacted with genomic calf thymus DNA, and the levels of 3-MeA (minor groove adduct) and N7-methylguanine (7-MeG, major groove adduct) formed were quantitated by reversed phase HPLC analysis, following previously published procedures.<sup>39</sup> The expected specific binding of **1** in the minor groove at A/T-rich regions would result in 3-MeA formation, while non-specific DNA methylation would primarily result in the formation of 7-MeG since the N7-guanine site is one of the most nucleophilic sites on DNA<sup>40–42</sup> and lies in the more accessible major groove of DNA. The methylation profile of **1** was compared to those of Me-lex, which contains the same DNA-binding core as **1** but lacks the ER-targeting estradiol moiety, and MMS, which has no DNA-binding component, and represents the methylating terminus of both Me-lex and **1**. Methylation at the O<sup>6</sup> position of guanine was not measured since it has been previously demonstrated that neither Me-lex,<sup>43</sup> nor MMS,<sup>44</sup> yield significant amounts of this adduct. Similarly, levels of N3-methylguanine were also not measured since it has been

shown that Me-lex produces only minor amounts of this adduct in comparison to 3-MeA.<sup>39</sup> Also, the effect of pre-treatment of calf thymus DNA with netropsin, a compound which inhibits DNA methylation in the minor groove at A/T rich regions,<sup>39</sup> on the DNA-methylating ability of **1**, Me-lex and MMS was also determined. The results obtained from these experiments are summarized in Table 1.

The major DNA adduct formed by **1** is the desired 3-MeA adduct. Of the total DNA adducts measured, **1** produces 86% 3-MeA and only 14% 7-MeG. A comparison of the relative amounts of 3-MeA and 7-MeG produced by **1**, MMS and Me-lex is shown in Figure 4. As can be seen from the figure, this minor groove selectivity of **1** is comparable to that of Me-lex, which produces 96% 3-MeA, and indicates that the alkyl estradiol component of **1** does not compromise its preference for the minor groove. By contrast, MMS, which lacks any DNA-binding component, produces predominantly (over 90%) the major groove 7-MeG adduct. The relative levels of 3-MeA and 7-MeG produced by MMS and Me-lex observed in these experiments are similar to those reported in literature.<sup>39,44</sup> Since the predominant adduct formed by **1** is 3-MeA, and since 7-MeG has been shown to be a benign adduct,<sup>39</sup> any biological consequence resulting from the interaction of **1** with cellular DNA is likely to be due to the formation of 3-MeA lesions.

In order to determine whether the 3-MeA adducts formed by **1** are produced in A/T rich regions, the inhibitory effect of netropsin on the methylation reactions was evaluated. Netropsin is a compound that also binds to the minor groove of DNA at A/T rich regions,<sup>45</sup> but it does so much more strongly (by over 2 orders of magnitude) than Me-lex<sup>22</sup> due to the fact that it is dicationic. When the DNA was pre-incubated with netropsin before treatment with the methylating agents, the levels of 3-MeA produced by **1** and Me-lex decreased by over 90% (see Table 1), thus confirming that the N3-adenine sites that are methylated by these two compounds are in A/T rich regions. By contrast, in the case of MMS, pre-treatment with netropsin decreased the levels of 3-MeA produced by 31%, indicating that only about a third of the N3-adenine sites methylated by MMS are at A/T rich regions.

The relative DNA-methylating potency of **1**, MMS and Me-lex was compared on an equimolar basis (500  $\mu$ M) and normalized against MMS adduct levels as shown in Table 2. This comparison



Scheme 1. Syntheses of compounds.

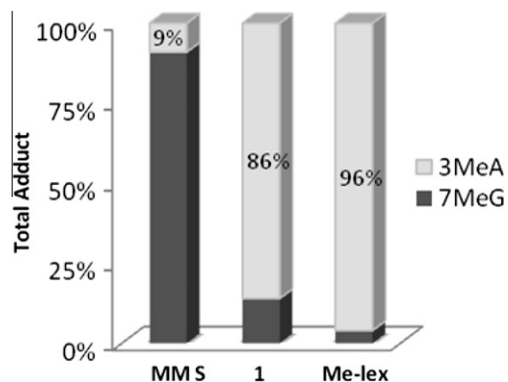
**Table 1**  
DNA adduct levels obtained upon reaction of compounds with calf thymus DNA<sup>a</sup>

Compound	Concn (μM)	Netropsin (μM)	Adduct level (μmol adduct/mol DNA)	
			3-MeA	7-MeG
<b>1</b>	50		1764 ± 17	280 ± 36
	50	100	167 ± 5	279 ± 43
MMS	5000		1799 ± 39	18,067 ± 76
	5000	100	1239 ± 88	18,935 ± 502
Me-lex	100		14,078 ± 224	496 ± 31
	100	100	657 ± 12	614 ± 67

<sup>a</sup> DNA (1 mM) was reacted with different methylating compounds, in the presence or absence of netropsin, for 24 h at room temperature in 10 mM sodium cacodylate buffer (pH 7.0) containing 10% DMSO.

shows that **1** produces 11 times as much total DNA adducts as MMS does, but is 3.6 times less potent than Me-lex. A comparison of only the major groove adduct (7-MeG) levels shows that both Me-lex and **1** produce 40–50% more 7-MeG than MMS.

None of the three compounds are expected to have specific binding interactions within the major groove of DNA, and it is therefore interesting that the larger molecules (**1** and Me-lex) containing the same methylating unit as MMS are more efficient major groove methylators. In the minor groove, **1** is about a 100 times more effective than MMS in producing this desired 3-MeA



**Figure 4.** A comparison of the ratios of 3-MeA and 7-MeG produced by MMS, **1** and Me-lex.

lesion, indicating that specific binding in the minor groove must be responsible for this dramatic increase in the levels of 3-MeA formed. The levels of 3-MeA produced by **1** are 25% of those produced by Me-lex, and this can be attributed to the probable decrease in the strength of binding of **1** in the narrow minor groove compared to Me-lex, probably due to its added bulk and



**Table 2**  
Relative DNA adduct levels<sup>a</sup>

	MMS	<b>1</b>	Me-lex
Total adducts (3-MeA + 7-MeG)	1	11	37
Major groove adduct (7-MeG)	1	1.5	1.4
Minor groove adduct (3-MeA)	1	98	391

<sup>a</sup> Compared on an equimolar basis (500  $\mu$ M) and normalized against MMS adduct levels.

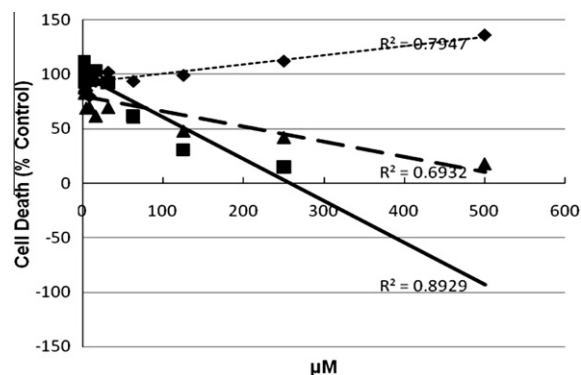
due to the flexibility of the alkyl chain that serves as the linker unit.

## 2.4. Estrogen receptor binding

In order to successfully target ER<sup>+</sup> cells, **1** has to exhibit reasonable binding to the ER protein. It has been reported that in most breast cancers there are between 3000 and 50,000 ER molecules per cell.<sup>32,33</sup> Taking into account the number of ER molecules per cell, and possible drug concentrations, it has been calculated that ER ligand conjugates must have a relative binding affinity (RBA) of at least 1%, when compared to the binding affinity of the natural ligand estradiol (taken to be 100%), in order to be effective. However, **1** is a reactive molecule, and could not be directly used to determine its ER-binding affinity since it may methylate nucleophilic sites on the ER during the binding assay. Hence the stable analog **2** (which is identical to **1** except for the absence of one oxygen atom at the end of the molecule far from the ER-binding estradiol unit) was used for these ER-binding studies. The binding interaction of **2** with the ER was determined using the Lanthascreen<sup>®</sup> TR-FRET Estrogen Receptor Alpha Competitive Binding Assay kit (Invitrogen). Using this assay, it was determined (Fig. 5) that the IC<sub>50</sub> value for estradiol was 0.57 nM, while that for **2** was 1.11 nM. Based on these values, the RBA of **2** was determined to be 51%, indicating that **2** is likely to have significant ER-binding. Therefore **1**, which would be expected to have almost identical ER-binding properties as **2**, would also have sufficient ER-binding in order to target ER<sup>+</sup> cells.

## 2.5. Biological assays

The toxicity of the different compounds was assessed in MCF-7 cells, a breast cancer cell line, that is, known to express the ER.<sup>46</sup> The cells were exposed to **1**, **2**, MMS and Me-lex for 16 h and the toxicity then determined by the MTT assay.<sup>47</sup> The results of these experiments are shown in Fig. 6. Compound **1** (EC<sub>50</sub> = 127  $\mu$ M) was the most toxic, followed by Me-lex (EC<sub>50</sub> = 214  $\mu$ M). MMS

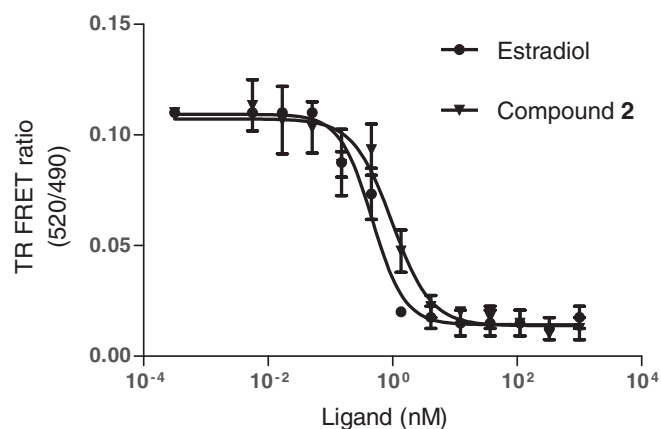


**Figure 6.** Toxicity of **1** (■), **2** (◆), and Me-lex (▲) in MCF-7 cells measured by MTT assay. Compound **1** was the most toxic (EC<sub>50</sub> = 127  $\mu$ M) followed by Me-lex (EC<sub>50</sub> = 214  $\mu$ M). Compound **2** was not toxic at the highest concentration tested (500  $\mu$ M).

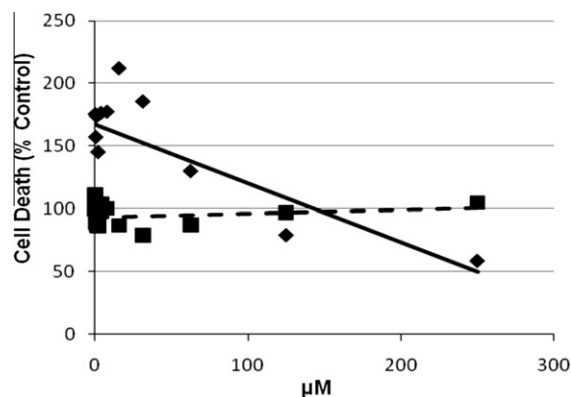
was much less toxic (data not shown), with an EC<sub>50</sub> concentration (2.75 mM) being more than 20 times higher than that of **1**. The toxicity of **1** could result from other mechanisms, such as ER-antagonism resulting in interference with normal ER function, or DNA-binding resulting in interference with normal genomic function, etc., and not necessarily due to DNA methylation. However, if any such alternate mechanisms play a role in the observed toxicity, analog **2** would be equally toxic to the cells. It was found that **2** was non-toxic to MCF-7 cells at the highest dose tested (500  $\mu$ M), thus confirming that methylation of a cellular component by **1** plays a role in its observed toxicity.

In order to assess the mediatory role of the ER in the toxicity of **1**, the effect of pre-treatment with a non-toxic dose of fulvestrant,<sup>48</sup> a known ER antagonist, on the toxicity of **1** in MCF-7 cells was determined. Fulvestrant binds to the ER with binding affinity similar to estradiol, and prevents ER translocation to the nucleus.<sup>49</sup> When MCF-7 cells were first treated with fulvestrant before the addition of **1**, no toxicity was seen (Fig. 7) at the highest concentration tested (250  $\mu$ M), thus demonstrating the central role of the ER in the mechanism of toxicity of **1**.

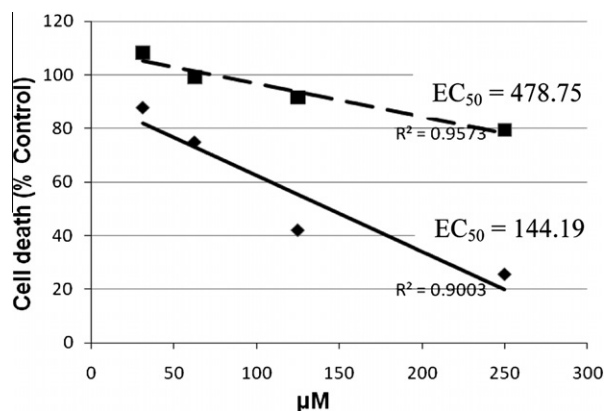
The toxicity of **1** could result from the methylation of non-DNA components of the cell. The ER protein itself could be an important target, since **1** has specific binding for the ER. In order to determine whether DNA methylation at A/T rich regions was involved in the toxicity of **1**, MCF-7 cells were first treated with netropsin before treatment with **1**, since experiments with calf thymus DNA had shown that netropsin can prevent the formation of 3-MeA adducts



**Figure 5.** TR-FRET ER $\alpha$  competitive binding assay.



**Figure 7.** Toxicity of **1** in MCF-7 cells in the absence (◆) and presence (■) of ER antagonist fulvestrant. Compound **1** was not toxic in MCF-7 cells pre-treated with fulvestrant.



**Figure 8.** Effect of netropsin on toxicity of **1** in MCF-7 cells, measured by MTT assay. There was a 3.3-fold decrease in toxicity in cells pre-treated with netropsin (■) compared to cells not treated with netropsin (◆).

**Table 3**  
EC<sub>50</sub> values

Compound	Cell type	Inhibitor	EC <sub>50</sub> (µM)
<b>1</b>	MCF-7	—	135 <sup>a</sup>
<b>1</b>	MCF-7	Netropsin	479
<b>1</b>	MCF-7	Fulvestrant	Not toxic <sup>b</sup>
<b>2</b>	MCF-7	—	Not toxic <sup>c</sup>
Me-lex	MCF-7	—	214
MMS	MCF-7	—	2750

<sup>a</sup> Average of values obtained from two experiments, using **1** from two different synthetic batches.

<sup>b</sup> Highest concentration tested: 250 µM.

<sup>c</sup> Highest concentration tested: 500 µM.

by **1**, and there is evidence that netropsin can attenuate 3-MeA formation by Me-lex in vivo.<sup>50</sup> This pre-exposure to netropsin resulted in a threefold decrease in the toxicity of **1** (Fig. 8), suggesting that 3-MeA formation is the primary reason for the cytotoxicity of **1** in MCF-7 cells.

A comparison of the EC<sub>50</sub> values (Table 3) obtained for the three compounds (**1**, Me-lex and MMS) shows that **1** is about 1.5 times as toxic as Me-lex, and about 20 times as toxic as MMS in MCF-7 cells. The 50% increase in lethality of **1** compared to Me-lex is noteworthy, since **1** produces only 25% of the lethal 3-MeA adducts that are produced by Me-lex, and strongly suggests that there must be improved delivery of **1** to the nucleus of MCF-7 cells relative to Me-lex.

### 3. Summary

In conclusion, a new compound that can target ER+ cells, and generate cytotoxic 3-MeA DNA lesions in these cells, has been developed. This compound, **1**, targets ER+ breast cancer cells due to its specific binding to the ER protein over-expressed in these cells, and destroys these cells due to the formation of 3-MeA adducts. Both ER-binding and site-specific DNA-binding are required for toxicity, and inhibition of the interaction of **1** with either biomolecule (DNA or ER) attenuates its toxicity. Experiments are currently in progress to test toxicity in other cell lines, and to determine selectivity for ER+ cells. The successful development of this compound demonstrates that this strategy can be used to target any cell-type that expresses a unique receptor or enzyme, and generate cytotoxic, non-mutagenic 3-MeA adducts in those cells. This strategy can lead to the development of drugs that offer clinical advantages over currently used alkylating agents and can

aid in the investigation of new mechanisms of therapy based upon autoimmune responses.

## 4. Experimental

### 4.1. Synthesis

**General:** All solvents and reagents were purchased from standard suppliers such as VWR International (West Chester, Pennsylvania), Sigma–Aldrich (Atlanta, Georgia) or Chem-Impex International (Wood Dale, Illinois), etc., and were of the highest grade available unless otherwise noted. Solvents and reagents are abbreviated as follows: DCM (dichloromethane); DIEA (*N,N*-diisopropylethylamine); DMAP (4-dimethylaminopyridine); DMF (*N,N*-dimethylformamide); DMSO (dimethyl sulfoxide); EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride); EtOAc (ethyl acetate); EtOH (ethanol); HOBT (1-hydroxybenzotriazole); MeOH (methanol); TEA (triethylamine); THF (tetrahydrofuran). Thin layer chromatography was performed on Silica Gel 50 F<sub>254</sub> (EMD Chemicals USA) and visualization was accomplished with a 254 nm UV light. Column chromatography was carried out using silica gel, 32–63 µm, 230–400 mesh (Dynamic Adsorbents Inc.). Rotary evaporations were carried out using a Buchi R-3000 or a Buchi R-114 rotary evaporator equipped with a Brinkmann model B-16 vacuum aspirator. Hydrogenations were performed using a Parr Hydrogenation Apparatus in a 500 mL Parr jar. All anhydrous reactions were carried out under positive pressure of nitrogen. Glassware used for anhydrous reactions was dried overnight at 110 °C or over a flame, assembled while still hot, and cooled to room temperature under nitrogen. Solvents and liquid reagents for anhydrous reactions were obtained in bottles with sure-seal caps and transferred by using oven-dried needles and glass syringes.

NMR spectra were recorded on a Bruker AMX 400 operating at 400.13 and 100.62 MHz for <sup>1</sup>H and <sup>13</sup>C, respectively. Deuterated (*d*<sub>6</sub>)-DMSO and deuterated chloroform were used as NMR solvents. Deuterated DMSO was obtained in sealed ampoules from Cambridge Isotope Laboratories, Inc (Andover, Massachusetts). The deuterated chloroform was obtained from Alfa Aesar. The spectra are reported in ppm and referenced to the DMSO peak (2.49 ppm for <sup>1</sup>H, 39.5 ppm for <sup>13</sup>C) or to chloroform peak (7.26 ppm for <sup>1</sup>H, 77 ppm for <sup>13</sup>C). Reported spin multiplicities are abbreviated as follows: s (singlet); br s (broad singlet); d (doublet); t (triplet); q (quartet); qu (quintet); m (multiplet); dd (doublet of doublets). Coupling constants are reported in hertz (Hz). All spectra were taken in DMSO unless otherwise mentioned. The samples were contained in 5 mm Pyrex glass NMR tubes obtained from Wilmad-LabGlass (Buena, New Jersey). High resolution mass spectrometry data was collected on a JEOL JMS-SX-102 High Resolution Mass Spectrometer.

#### 4.1.1. (7*R*,13*S*,17*S*)-7-(6-Aminohexyl)-17-((*tert*-butyldimethylsilyloxy)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthren-3-ol) (**12**)

Compound **12** was synthesized according to previously reported procedures.<sup>37</sup> During the final step of the synthesis following reported procedures, the aqueous work-up resulted in the mono-protected **12**, instead of the bis-protected compound reported in literature. Mp = 67–69 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.10 (d, *J* = 8.4 Hz, 1H), 6.27 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 3.64 (m, 1H), 3.49 (q, *J* = 7.2 Hz, 2H), 3.26 (br s, 3H), 2.85 (d, 1H), 2.69 (t, 2H), 2.25 (m, 2H), 1.95 (m, 1H), 1.82 (m, 1H), 1.72 (m, 1H), 1.60–1.10 (m, 15H), 1.05–0.95 (m, 1H), 0.85 (s, 9H), 0.72 (s, 3H), 0.02 (s, 6H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 154.46, 136.92, 131.12, 126.94, 116.36, 113.17, 81.86, 52.78, 46.09, 43.73, 42.08, 41.72,

38.25, 37.36, 34.67, 33.31, 32.64, 30.95, 29.68, 28.05, 27.40, 26.81, 25.89, 25.44, 22.81, 18.14, 11.41, 7.86, –4.44, –4.76. HRMS (ESI)  $m/z$  for  $C_{30}H_{51}NO_2Si$   $[M+H]^+$  calcd 486.3762, found 486.3765.

#### 4.1.2. 3-(Methylsulfonyl)propanoic acid (**13**)

To 3-methylthiopropionic acid (14.29 g, 118.9 mmol) in a 250 mL flask was added 57 mL acetic anhydride and 57 mL acetic acid and the mixture cooled in an ice bath and stirred for 30 min. Then 50%  $H_2O_2$  (36 mL) was added 1 mL at a time in 5 mL installments every 15 min. Upon completion of the reaction as indicated by NMR, a trace of  $MnO_2$  was added to quench excess  $H_2O_2$ , and the mixture stirred for 2 h. The solution was filtered through Celite, the organic solvents removed with a rotary evaporator and the residue was placed under vacuum for 24 h to yield **13** as a white solid (18.05 g, 99% yield). Mp = 100–102 °C.  $^1H$  NMR data:  $\delta$  12.59 (br s, 1H), 3.33 (t,  $J$  = 7.2 Hz, 2H), 2.99 (s, 3H), 2.68 (t,  $J$  = 7.2 Hz, 2H).  $^{13}C$  NMR:  $\delta$  172.72, 50.35, 41.15, 28.03. HRMS (ESI)  $m/z$  for  $C_4H_8O_4S$   $[M+Na]^+$  calcd 175.0036, found 175.0035.

#### 4.1.3. 2,2,2-Trichloro-1-(1-methyl-4-nitro-1H-pyrrol-2-yl)ethanone (**3**)

Compound **3** was synthesized according to previously reported procedures.<sup>36</sup> Mp = 127–129 °C. TLC (1:1 EtOAc/hexane)  $R_f$  = 0.56.  $^1H$  NMR:  $\delta$  8.56 (d,  $J$  = 1.7 Hz, 1H), 7.78 (d,  $J$  = 1.7 Hz, 1H), 3.98 (s, 3H).  $^{13}C$  NMR:  $\delta$  173.30, 134.73, 133.09, 121.09, 116.82, 95.02, 79.44. HRMS (ESI)  $m/z$  for  $C_7H_5Cl_3N_2O_3$   $[M+H]^+$  calcd 270.9439, found 270.9439.

#### 4.1.4. Ethyl 4-(1-methyl-4-nitro-1H-pyrrole-2-carboxamido)butanoate (**4a**)

Compound **3** (10.1 g, 0.037 mol) was placed in a 500 mL round bottom flask and dissolved in 75 mL of dry EtOAc. Ethyl 4-aminobutyrate hydrochloride (9.35 g, 0.058 mol) was added to the flask. Dry TEA (15.05 mL, 0.108 mol) was added to the flask dropwise over 1 h. The reaction mixture was stirred under a nitrogen atmosphere. When TLC (EtOAc) indicated that **3** was absent, the solution was filtered by vacuum filtration to remove the white solid precipitate that was present. The filtrate was transferred to a 500 mL separatory funnel, and the organic layer was washed with 1 M HCl (2  $\times$  100 mL), DI  $H_2O$  (1  $\times$  100 mL), 5%  $NaHCO_3$  (2  $\times$  100 mL), and DI  $H_2O$  (1  $\times$  100 mL). The organic layer was dried over anhydrous magnesium sulfate, and the solvent was removed by rotary evaporation. The residue was placed under vacuum for 24 h to yield **4a** as a yellow solid (8.44 g, 80% yield). Mp = 63–65 °C. TLC (1:1 EtOAc/hexane)  $R_f$  = 0.25;  $^1H$  NMR ( $CDCl_3$ )  $\delta$  7.54 (d,  $J$  = 1.6 Hz, 1H), 7.08 (d,  $J$  = 1.6 Hz, 1H), 6.54–6.50 (br s, 1H), 4.16 (q,  $J$  = 7.2 Hz, 2H), 3.99 (s, 3H), 3.44 (q,  $J$  = 7.2 Hz, 2H), 2.44 (t,  $J$  = 6.8 Hz, 2H), 1.94 (qu,  $J$  = 6.8 Hz, 2H), 1.26 (t,  $J$  = 7.2 Hz, 3H).  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  173.72, 160.54, 134.78, 126.72, 126.49, 107.08, 60.72, 39.13, 37.84, 31.90, 24.35, 14.13. HRMS (ESI)  $m/z$  for  $C_{12}H_{17}N_3O_5$   $[M+H]^+$  calcd 284.1241, found 284.1240.

#### 4.1.5. tert-Butyl 4-(1-methyl-4-nitro-1H-pyrrole-2-carboxamido)butanoate (**4b**)

Compound **3** (1.350 g, 4.97 mmol) was placed in a 100 mL round bottom flask and dissolved in 20 mL of dry EtOAc.  $\gamma$ -aminobutyric acid *t*-butyl ester hydrochloride (1.083 g, 5.53 mmol) was added to the flask. Dry TEA (2.0 mL, 14.3 mmol) was added to the flask dropwise over 1 h and the reaction worked up exactly as for **4a** to yield **4b** as a yellow solid (1.486 g, 96% yield). Mp = 89–90 °C. TLC (EtOAc)  $R_f$  = 0.66.  $^1H$  NMR data:  $\delta$  8.39 (t,  $J$  = 5.6 Hz, 1H), 8.12 (d,  $J$  = 1.6 Hz, 1H), 7.43 (d,  $J$  = 1.6 Hz, 1H), 3.90 (s, 3H), 3.19 (q,  $J$  = 7.2 Hz, 5.6 Hz, 2H), 2.24 (t,  $J$  = 7.2 Hz, 2H), 1.70 (qu,  $J$  = 7.2 Hz, 2H), 1.39 (s, 9H).  $^{13}C$  NMR data:  $\delta$  172.45, 160.27, 134.19, 128.29, 126.87, 107.75, 80.02, 38.39, 37.84, 32.68, 28.21, 24.96.

#### 4.1.6. Ethyl 4-(1-methyl-4-(1-methyl-4-nitro-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanoate (**5a**)

To **4a** (8.4 g, 0.029 mol) in 50 mL of 95% EtOH in a Parr jar was added 1 g of wet 10% palladium on activated carbon. The jar was then pressurized to 70 psi with hydrogen, and shaken. When TLC (EtOAc) indicated that **4a** was no longer present, the solution was filtered over Celite, which was washed with EtOH (2  $\times$  50 mL). The combined solutions were concentrated by rotary evaporation and the remaining liquid placed under vacuum for 24 h. The resultant dark oil was dissolved in 50 mL of dry EtOAc, and **3** (8.86 g, 0.033 mol) was added. The solution was stirred under a nitrogen atmosphere for 48 h, during which time a yellow precipitate formed. The precipitate was collected by vacuum filtration to yield **5a** as a bright yellow solid (8.42 g, 70% yield). Mp = 151–152 °C. TLC (EtOAc)  $R_f$  = 0.54.  $^1H$  NMR:  $\delta$  10.23 (s, 1H), 8.13 (d,  $J$  = 1.6 Hz, 1H), 8.07 (t,  $J$  = 5.6 Hz, 1H), 7.58 (d,  $J$  = 1.6 Hz, 1H), 7.20 (d,  $J$  = 1.6 Hz, 1H), 6.87 (d,  $J$  = 1.6 Hz, 1H), 4.03 (q,  $J$  = 7.2 Hz, 2H), 4.00 (s, 3H), 3.81 (s, 3H), 3.19 (q,  $J$  = 6 Hz, 2H), 2.31 (t,  $J$  = 6 Hz, 2H), 1.74 (qu,  $J$  = 6 Hz, 2H), 1.16 (t,  $J$  = 7.2 Hz, 3H).  $^{13}C$  NMR:  $\delta$  173.15, 161.67, 157.31, 134.27, 128.59, 126.75, 123.64, 121.83, 118.45, 108.01, 104.50, 60.20, 38.21, 37.91, 36.45, 31.51, 25.16, 14.51. HRMS (ESI)  $m/z$  for  $C_{18}H_{23}N_5O_6$   $[M+H]^+$  calcd 406.1721, found 406.1721.

#### 4.1.7. tert-Butyl 4-(1-methyl-4-(1-methyl-4-nitro-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanoate (**5b**)

To **4b** (0.760 g, 2.5 mmol) in 25 mL of 95% EtOH in a Parr jar was added 0.180 g of wet 10% palladium on activated carbon. The jar was then pressurized to 70 psi with hydrogen, and shaken. When TLC (6:1 EtOAc/MeOH) indicated that **4b** was no longer present, the solution was filtered over Celite which was washed with ethanol (2  $\times$  50 mL). The combined solutions were concentrated by rotary evaporation and the remaining liquid placed under vacuum for 24 h. The resultant dark oil was dissolved in 20 mL of dry EtOAc, and **3** (0.611 g, 2.75 mmol) was added. The solution was stirred under a nitrogen atmosphere for 48 h. The solvent was removed by rotary evaporation and the residue purified by flash column chromatography (5:2 EtOAc/hexane) to yield **5b** as a yellow solid (0.574 g, 53% yield). Mp = 154–157 °C. TLC (5:2 EtOAc/hexane)  $R_f$  = 0.41.  $^1H$  NMR:  $\delta$  10.24 (s, 1H), 8.19 (d,  $J$  = 1.6 Hz, 1H), 8.08 (t,  $J$  = 5.6 Hz, 1H), 7.58 (d,  $J$  = 1.6 Hz, 1H), 7.20 (d,  $J$  = 1.6 Hz, 1H), 6.86 (d,  $J$  = 1.6 Hz, 1H), 3.95 (s, 3H), 3.81 (s, 3H), 3.17 (q,  $J$  = 7.2 Hz, 5.6 Hz, 2H), 2.22 (t,  $J$  = 7.2 Hz, 2H), 1.69 (qu,  $J$  = 7.2 Hz, 2H), 1.39 (s, 9H).  $^{13}C$  NMR:  $\delta$  172.53, 161.60, 157.29, 134.23, 128.70, 126.76, 123.63, 121.78, 118.41, 108.00, 104.47, 79.97, 38.18, 37.95, 36.50, 32.78, 28.23, 25.32. HRMS (ESI)  $m/z$  for  $C_{20}H_{27}N_5O_6$   $[M+Na]^+$  calcd 456.1854, found 456.1857.

#### 4.1.8. Ethyl 4-(4-(4-acrylamido-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)butanoate (**6**)

To **5a** (0.310 g, 0.764 mmol) in 25 mL of 95% EtOH was added 0.180 g of wet 10% palladium on activated carbon. The jar was then pressurized to 70 psi with hydrogen, and shaken. When TLC (EtOAc) indicated that **5a** was no longer present, the solution was filtered over Celite, which was washed with ethanol (2  $\times$  50 mL). The combined solutions were concentrated by rotary evaporation and the remaining liquid placed under vacuum for 24 h. The resultant dark oil was dissolved in 5 mL of anhydrous THF, and stirred under a nitrogen atmosphere in a dry ice/acetone bath at –40 °C for 15 min. DIEA (0.4 mL, 2.3 mmol) was added and the reaction stirred for 30 min at –40 °C after which acryloyl chloride (0.067 mL, 0.81 mmol) was added and the flask covered with aluminum foil to protect it from light. The reaction mixture was stirred under a nitrogen atmosphere for 24 h, concentrated by rotary evaporation and the resulting oil re-dissolved in 100 mL of EtOAc and washed

with DI H<sub>2</sub>O (2 × 100 mL). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed by rotary evaporation. The residue was protected from light by aluminum foil and placed under vacuum for 24 h to yield **6** as a brown solid (0.230 g, 65% yield). Mp = 87–90 °C. TLC (6:1 EtOAc/MeOH) *R<sub>f</sub>* = 0.55. <sup>1</sup>H NMR: δ 10.10 (s, 1H), 9.89 (s, 1H), 8.03 (t, *J* = 5.6 Hz, 1H), 7.26 (d, *J* = 1.8 Hz, 1H), 7.18 (d, *J* = 1.8 Hz, 1H), 6.91 (d, *J* = 1.8 Hz, 1H), 6.85 (d, *J* = 1.8 Hz, 1H), 6.37 (dd, *J* = 17 Hz, *J* = 10.2 Hz, 1H), 6.20–6.15 (dd, *J* = 17 Hz, *J* = 2.1 Hz, 1H), 5.66 (dd, *J* = 10.2 Hz, 2.1 Hz, 1H), 4.04 (q, *J* = 7.2 Hz, 2H), 3.84 (s, 3H), 3.79 (s, 3H), 3.18 (q, *J* = 6 Hz, 5.6 Hz, 2H), 2.31 (t, *J* = 7.2 Hz, 2H), 1.73 (qu, *J* = 7.2 Hz, 6 Hz, 2H), 1.17 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR: δ 173.18, 162.09, 161.75, 158.74, 131.95, 126.04, 123.47, 123.33, 122.46, 122.15, 118.89, 118.32, 104.63, 104.41, 60.22, 38.16, 36.65, 36.42, 31.50, 25.17, 14.57. HRMS (ESI) *m/z* for C<sub>21</sub>H<sub>27</sub>N<sub>5</sub>O<sub>5</sub> [M+H]<sup>+</sup> calcd 430.2085, found 430.2087.

#### 4.1.9. 4-(4-(4-Acrylamido-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)butanoic acid (**7**)

Compound **6** (0.800 g, 1.8 mmol) was dissolved in 12 mL of acetone in a 100 mL round bottom flask. A solution of NaOH (0.450 g, 11 mmol) in 3 mL of DI H<sub>2</sub>O was added in one portion and the stirred reaction covered with aluminum foil to protect it from light. When TLC (4.5:2.5 EtOAc/MeOH) indicated that **6** was no longer present, the reaction mixture was concentrated to 1 mL by rotary evaporation, 2 mL of cold DI H<sub>2</sub>O added and the flask cooled in an ice bath for 15 min. The mixture was acidified to pH 1 with concentrated HCl, causing a yellow precipitate to form. After the flask was cooled in an ice bath for an additional 30 min, the precipitate was collected by vacuum filtration to yield **7** as a yellow solid (0.610 g, 83% yield). Mp = 71–76 °C. TLC (1:1 EtOAc/MeOH) *R<sub>f</sub>* = 0.51. <sup>1</sup>H NMR: δ 12.09 (br s, 1H), 10.19 (s, 1H), 9.90 (s, 1H), 8.1–8.0 (br s, 1H), 7.26 (d, *J* = 1.6 Hz, 1H), 7.18 (d, *J* = 1.6 Hz, 1H), 6.93 (d, *J* = 1.6 Hz, 1H), 6.86 (d, *J* = 1.6 Hz, 1H), 6.40 (dd, *J* = 17 Hz, 10 Hz, 1H), 6.16 (dd, *J* = 17 Hz, 2 Hz, 1H), 5.63 (dd, *J* = 10 Hz, 2 Hz, 1H), 3.83 (s, 3H), 3.78 (s, 3H), 3.17 (m, 2H), 2.24 (t, *J* = 7.2 Hz, 2H), 1.70 (qu, *J* = 7.2 Hz, 2H). <sup>13</sup>C NMR: δ 174.77, 162.15, 161.78, 158.79, 132.01, 125.94, 123.50, 123.41, 122.47, 122.18, 118.92, 118.34, 104.69, 104.50, 38.30, 36.63, 36.39, 31.64, 25.22. HRMS (ESI) *m/z* for C<sub>19</sub>H<sub>23</sub>N<sub>5</sub>O<sub>5</sub> [M+H]<sup>+</sup> calcd 402.1772, found 402.1772.

#### 4.1.10. 4-Acrylamido-N-((5-((4-((6-((7R,13S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenathren-7-yl)hexyl)amino)-4-oxobutyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)-1-methyl-1H-pyrrole-2-carboxamide (**8**)

To compound **7** (0.100 g, 0.25 mmol) in an oven-dried round bottom flask was added EDCI (0.143 g, 0.7 mmol), DMAP (0.108 g, 0.8 mmol), HOBT (0.135 g, 1.0 mmol) and anhydrous DMF (4 mL). The mixture was stirred under a nitrogen atmosphere for 30 min until all solids dissolved. Compound **12** (0.109 g, 0.23 mmol) was then added and the reaction stirred under a nitrogen atmosphere for 24 h, after which 40 mL of cold 1 M HCl was added causing a white precipitate to form. The solution was stirred for an additional 2 h, and the precipitate collected by vacuum filtration. The precipitate was dissolved in a minimal volume of DMF, and 40 mL of cold 5% NaHCO<sub>3</sub> was added, causing a white precipitate to form. The mixture was stirred for 30 min, and the precipitate collected by vacuum filtration to yield **8** as a pale white solid (0.162 g, 75% yield). Mp = 172–175 °C. TLC (6:1 EtOAc/MeOH) *R<sub>f</sub>* = 0.61. <sup>1</sup>H NMR: δ 10.13 (s, 1H), 9.91 (s, 1H), 9.00 (s, 1H), 8.02 (t, *J* = 5.6 Hz, 1H), 7.78 (t, *J* = 5.6 Hz, 1H), 7.27 (s, 1H), 7.19 (s, 1H), 7.04 (d, *J* = 7.6 Hz, 1H), 6.92 (s, 1H), 6.85 (s, 1H), 6.49 (d, *J* = 7.6 Hz, 1H), 6.41 (s, 1H), 6.37 (dd, *J* = 17 Hz, 10 Hz, 1H), 6.18 (dd, *J* = 17 Hz, 1.6 Hz, 1H), 5.66 (dd, *J* = 10 Hz, 1.6 Hz, 1H), 4.50 (d, *J* = 4.4 Hz, 1H), 3.84 (s, 3H), 3.79 (s, 3H), 3.53 (m, 1H), 3.14 (q,

*J* = 6 Hz, 2H), 2.99 (q, *J* = 6 Hz, 2H), 2.73 (d, 1H), 2.62 (d, 1H), 2.24 (d, 1H), 2.16 (t, 1H), 2.07 (t, *J* = 7.2 Hz, 2H), 1.87 (m, 1H), 1.77 (d, 1H), 1.66 (m, 3H), 1.47 (m, 2H), 1.40–1.10 (m, 15H), 0.95–0.80 (m, 1H), 0.66 (s, 3H). <sup>13</sup>C NMR: δ 172.08, 162.09, 161.69, 158.74, 155.42, 136.48, 131.95, 130.10, 127.13, 126.07, 123.47, 123.39, 122.44, 122.14, 118.89, 118.29, 116.23, 113.32, 104.56, 104.41, 80.56, 46.44, 43.42, 42.20, 40.4 (under solvent peak), 38.91, 38.68, 38.23, 37.22, 36.66, 36.44, 34.59, 33.56, 33.21, 30.33, 29.64, 28.03, 27.53, 27.02, 26.13, 25.59, 22.74, 11.78. HRMS (ESI) *m/z* for C<sub>43</sub>H<sub>58</sub>N<sub>6</sub>O<sub>6</sub> [M+H]<sup>+</sup> calcd 755.4491, found 755.4491.

#### 4.1.11. 3-((5-((4-((6-((7R,13S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenathren-7-yl)hexyl)amino)-4-oxobutyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamoyl)-1-methyl-1H-pyrrole-2-carboxamide (**9**)

To compound **8** (0.090 g, 0.12 mmol) dissolved in 8 mL of 95% EtOH in a round bottom flask was added NaHSO<sub>3</sub> (0.087 g, 0.9 mmol) dissolved in 2 mL of DI H<sub>2</sub>O. The pH was adjusted to 8 with 5% NaOH and the reaction mixture refluxed until TLC (6:1 EtOAc/MeOH) indicated that **8** was no longer present. The reaction mixture was then concentrated to 1 mL by rotary evaporation and 2 mL of cold DI H<sub>2</sub>O was added. After cooling in an ice bath for 15 min., the mixture was acidified to pH 1 with concentrated HCl, causing a yellow precipitate to form. The flask was cooled in an ice bath for an additional 30 min, after which the precipitate was collected by vacuum filtration to yield **9** as a pale yellow solid (0.095 g, 95% yield). Mp = 220–224 °C. TLC (1:1 EtOAc/MeOH) *R<sub>f</sub>* = 0.68. <sup>1</sup>H NMR: δ 9.96 (s, 1H), 9.85 (s, 1H), 7.99 (t, *J* = 5.4 Hz, 1H), 7.77 (t, *J* = 5.4 Hz, 1H), 7.17 (s, 1H), 7.15 (s, 1H), 7.04 (d, *J* = 8.4 Hz, 1H), 6.85 (s, 2H), 6.49 (d, *J* = 8.4 Hz, 1H), 6.42 (s, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.14 (q, *J* = 6 Hz, 2H), 2.99 (q, *J* = 6 Hz, 2H), 2.73 (d, 1H), 2.69 (m, 1H), 2.62–2.53 (m, 2H), 2.23 (d, 1H), 2.17 (t, 1H), 2.07 (t, *J* = 7.2 Hz, 2H), 1.87 (m, 1H), 1.78 (d, 1H), 1.71–1.62 (m, 3H), 1.47 (m, 2H), 1.40–1.10 (m, 15H), 0.95–0.80 (m, 1H), 0.66 (s, 3H). <sup>13</sup>C NMR: δ 172.07, 168.93, 161.69, 158.81, 155.40, 136.43, 130.07, 127.08, 123.35, 123.08, 122.50, 118.53, 118.21, 116.21, 113.30, 104.56, 104.33, 80.52, 47.99, 46.41, 43.39, 42.18, 40.85, 40.48, 38.85, 38.63, 38.20, 37.19, 36.49, 36.35, 34.57, 33.52, 33.18, 32.91, 30.30, 29.59, 27.98, 27.50, 26.97, 26.08, 25.55, 22.71, 11.74. HRMS (ESI) *m/z* for C<sub>43</sub>H<sub>60</sub>N<sub>6</sub>O<sub>9</sub>S [M+H]<sup>+</sup> calcd 837.4215, found 837.4218.

#### 4.1.12. Methyl 3-((5-((4-((6-((7R,13S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenathren-7-yl)hexyl)amino)-4-oxobutyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamoyl)-1-methyl-1H-pyrrole-2-carboxamide (**1**)

Compound **9** (0.015 g, 0.02 mmol) was placed into an oven-dried round bottom flask with a reflux condenser and the system cooled to room temperature under a dry nitrogen atmosphere. Anhydrous THF (3 mL) was added and the mixture was heated to 60 °C with stirring until the solid dissolved. 1-methyl-3-(*p*-tolyl)-triaz-1-ene (0.030 g, 0.20 mmol) was added and the reaction stirred at reflux, while monitored by TLC (9:1 DCM/MeOH). After 1 h, TLC indicated that further formation of the desired product ceased, and the reaction was cooled to room temperature. The solution was purified by flash column chromatography (9:1 DCM/MeOH) to yield **1** as a white solid (0.003 g, 20% yield). Mp = 225–227 °C. TLC (9:1 DCM/MeOH) *R<sub>f</sub>* = 0.32. <sup>1</sup>H NMR: δ 10.05 (s, 1H), 9.86 (s, 1H), 8.97 (s, 1H), 7.99 (t, *J* = 6 Hz, 1H), 7.76 (t, *J* = 6 Hz, 1H), 7.17 (d, *J* = 1.8 Hz, 2H), 7.04 (d, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 1.8 Hz, 1H), 6.84 (d, *J* = 1.8 Hz, 1H), 6.49 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H), 6.41 (d, *J* = 2.4 Hz, 1H), 4.49 (s, *J* = 4.8 Hz, 1H), 3.86 (s, 3H), 3.83 (s, 3H), 3.79 (s, 3H), 3.61 (t, *J* = 7.2 Hz, 2H), 3.53 (t, 1H), 3.14 (q, *J* = 6 Hz, 2H), 2.99 (q, *J* = 6 Hz, 2H), 2.73 (m, 2H), 2.59 (d, 1H), 2.26 (d, 1H), 2.18 (t, 1H), 2.07 (t, *J* = 7.2 Hz, 2H), 1.88 (m, 1H), 1.78 (d, 1H),



1.67 (m, 3H), 1.47 (m, 2H), 1.40–1.10 (m, 15H), 0.95–0.80 (m, 1H), 0.66 (s, 3H).  $^{13}\text{C}$  NMR: 172.54, 166.49, 162.15, 159.21, 155.88, 136.94, 130.58, 127.58, 123.88, 122.90, 122.55, 119.08, 118.75, 116.70, 113.78, 105.05, 104.75, 81.02, 57.83, 46.91, 44.95, 43.88, 42.67, 40.98, 39.35, 39.14, 38.69, 37.69, 37.08, 36.87, 35.07, 34.02, 33.67, 31.35, 30.80, 30.09, 28.47, 27.99, 27.46, 26.58, 26.05, 24.49, 23.20, 12.23. HRMS (ESI)  $m/z$  for  $\text{C}_{44}\text{H}_{62}\text{N}_6\text{O}_9\text{S}$   $[\text{M}+\text{H}]^+$  calcd 850.4299, found 850.4293.

#### 4.1.13. *tert*-Butyl 4-(1-methyl-4-(1-methyl-4-(3-(methylsulfonyl)propanamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanoate (**10**)

To **5b** (0.550 g, 1.2 mmol) in 20 mL of 95% EtOH in a Parr jar was added 0.100 g of wet 10% palladium on activated carbon. The jar was then pressurized to 70 psi with hydrogen, and shaken. When TLC (6:1 EtOAc/MeOH) indicated that **5b** was no longer present, the solution was filtered over Celite which was washed with ethanol ( $2 \times 50$  mL). The combined solutions concentrated by rotary evaporation and the remaining liquid placed under vacuum for 24 h, resulting in a dark oil to which was added EDCI (0.143 g, 0.7 mmol), DMAP (0.108 g, 0.8 mmol), HOBT (0.135 g, 1.0 mmol) and anhydrous DMF (6 mL). The mixture stirred under a nitrogen atmosphere for 30 min until all solids dissolved at which time **13** (0.566 g, 3.7 mmol) was added. The mixture was stirred for 48 h, diluted with 100 mL of DCM and washed with 1 M HCl ( $2 \times 100$  mL), DI  $\text{H}_2\text{O}$  ( $1 \times 100$  mL), 5%  $\text{NaHCO}_3$  ( $2 \times 100$  mL), and then with DI  $\text{H}_2\text{O}$  ( $1 \times 100$  mL). The organic layer was dried over anhydrous magnesium sulfate, the solvent removed by rotary evaporation and the residue placed under vacuum for 24 h to yield **10** as a brown solid (0.419 g, 63% yield). Mp = 170–174 °C. TLC (6:1 DCM:MeOH)  $R_f$  = 0.76.  $^1\text{H}$  NMR:  $\delta$  10.07 (s, 1H), 9.87 (s, 1H), 8.02 (t,  $J$  = 5.6 Hz, 1H), 7.17 (t,  $J$  = 1.6 Hz, 2H), 6.86 (t,  $J$  = 1.6 Hz, 2H), 3.82 (s, 3H), 3.79 (s, 3H), 3.40 (t,  $J$  = 7.6 Hz, 2H), 3.16 (q,  $J$  = 6 Hz, 2H), 3.01 (s, 3H), 2.73 (t,  $J$  = 7.6 Hz, 2H), 2.22 (t,  $J$  = 7 Hz, 2H), 1.68 (qu,  $J$  = 7 Hz, 2H), 1.41 (s, 9H).  $^{13}\text{C}$  NMR:  $\delta$  172.56, 166.56, 161.74, 158.76, 123.34, 123.30, 122.44, 122.13, 118.64, 118.30, 104.66, 104.30, 79.99, 50.20, 38.16, 36.61, 36.41, 32.79, 28.66, 28.23, 25.33.

#### 4.1.14. 4-(1-Methyl-4-(1-methyl-4-(3-(methylsulfonyl)propanamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanoic acid (**11**)

Compound **10** (0.100 g, 0.2 mmol) and formic acid (10 mL) was added to a 50 mL round bottom flask and stirred. When TLC (5:2 EtOAc/MeOH) indicated that **10** was no longer present, the reaction was diluted with 20 mL of DCM, concentrated by rotary evaporation and the residue was placed under vacuum for 24 h to yield **11** as a white solid (0.09 g, 98% yield). Mp = 187–189 °C. TLC (1:1 EtOAc/MeOH)  $R_f$  = 0.53.  $^1\text{H}$  NMR:  $\delta$  12.06 (s, 1H), 10.07 (s, 1H), 9.88 (s, 1H), 8.04 (t,  $J$  = 5.6 Hz, 1H), 7.17 (q,  $J$  = 1.6 Hz, 2H), 6.86 (q,  $J$  = 1.6 Hz, 2H), 3.83 (s, 3H), 3.79 (s, 3H), 3.41 (t,  $J$  = 7.6 Hz, 2H), 3.17 (q,  $J$  = 6 Hz, 2H), 3.01 (s, 3H), 2.73 (t,  $J$  = 7.6 Hz, 2H), 2.23 (t,  $J$  = 7 Hz, 2H), 1.69 (qu,  $J$  = 7 Hz, 2H).  $^{13}\text{C}$  NMR:  $\delta$  174.77, 166.54, 161.75, 158.75, 123.38, 123.29, 122.44, 122.14, 118.63, 118.30, 104.63, 104.29, 50.21, 40.88, 38.28, 36.62, 36.41, 31.61, 28.67, 25.21.

#### 4.1.15. *N*-4-((6-((7R,13S,17S)-3,17-Dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[*a*]phenathren-7-yl)hexyl)amino)-4-oxobutyl)-1-methyl-4-(1-methyl-4-(3-(methylsulfonyl)propanamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamide (**2**)

To a mixture of **11** (0.261 g, 0.542 mmol), EDCI (0.208 g, 1.08 mmol), HOBT (0.227 g, 1.68 mmol), DMAP (0.160 g, 1.31 mmol), and compound **12** (0.263 g, 0.541 mmol) in a 50 mL round bottom flask was added anhydrous DMF (6 mL) and the mixture stirred under a nitrogen atmosphere for 48 h at which time 100 mL of cold 1 M HCl was added, resulting in a white precipitate.

The mixture was then further stirred for 72 h, after which the precipitate was collected by vacuum filtration. The precipitate was redissolved in a minimum volume of DMF and 100 mL of cold 5%  $\text{NaHCO}_3$  added to the flask, causing a white precipitate to form. The solution was stirred for 1 h, and the precipitate collected by vacuum filtration to yield **2** as a white solid (0.353 g, 78% yield). Mp = 239–241 °C. TLC (5:2 EtOAc/MeOH)  $R_f$  = 0.60.  $^1\text{H}$  NMR data:  $\delta$  10.07 (s, 1H), 9.88 (s, 1H), 8.97 (br s, 1H), 7.99 (t,  $J$  = 5.6 Hz, 1H), 7.77 (t,  $J$  = 5.6 Hz, 1H), 7.17 (d,  $J$  = 1.6 Hz, 2H), 7.04 (d,  $J$  = 8.4 Hz, 1H), 6.86 (d,  $J$  = 1.6 Hz, 1H), 6.84 (d,  $J$  = 1.6 Hz, 1H), 6.49 (dd,  $J$  = 8.4 Hz, 2.4 Hz, 1H), 6.41 (d,  $J$  = 2.4 Hz, 1H), 4.49 (d,  $J$  = 4.4 Hz, 1H), 3.83 (s, 3H), 3.79 (s, 3H), 3.53 (t, 1H), 3.39 (t,  $J$  = 7.2 Hz, 2H), 3.14 (q,  $J$  = 6.4 Hz, 2H), 3.01 (s, 3H), 2.99 (m, 2H), 2.73 (m, 2H), 2.59 (d, 1H), 2.25 (d, 1H), 2.17 (t, 1H), 2.07 (t, 7.6 Hz, 2H), 1.90 (m, 1H), 1.78 (d, 1H), 1.72–1.62 (m, 3H), 1.48 (m, 2H), 1.40–1.10 (m, 15H), 0.95–0.80 (m, 1H), 0.66 (s, 3H).  $^{13}\text{C}$  NMR:  $\delta$  172.07, 166.53, 161.68, 161.68, 158.74, 155.42, 136.48, 130.10, 127.13, 123.38, 123.28, 122.44, 122.13, 118.61, 118.27, 116.23, 113.31, 104.56, 104.26, 80.56, 50.19, 46.44, 43.42, 42.19, 40.87, 38.88, 38.66, 38.23, 37.22, 36.63, 36.43, 34.59, 33.55, 33.21, 30.33, 29.64, 28.65, 28.03, 27.53, 27.02, 26.28, 26.13, 25.59, 22.74, 11.78. HRMS (ESI)  $m/z$  for  $\text{C}_{44}\text{H}_{62}\text{N}_6\text{O}_8\text{S}$   $[\text{M}+\text{H}]^+$  calcd 834.4350, found 843.4341.

## 4.2. Analytical HPLC

The DNA-methylating compounds were reacted with 1 mM calf thymus DNA in 10 mM cacodylate buffer (pH 7.0) containing 10% DMSO for 24 h in the absence or presence of 100  $\mu\text{M}$  netropsin. The DNA was then subjected to neutral thermal hydrolysis (90 °C for 15 min) to liberate 7-MeG and 3-MeA and the apurinic DNA removed by precipitation after cooling, with the addition of cold 0.1 N HCl.<sup>44</sup> The supernatant containing the methylated purines was collected and analyzed on reverse phase HPLC using UV detection (270 nm). Analytical HPLC was performed on a Sonoma C<sub>18</sub>2 reverse phase 5  $\mu$ , 100 Å, 25 cm  $\times$  4.6 mm column. The mobile phase used was a 0.1 M NaOAc buffer with 4% methanol at a pH of 5.

## 4.3. Estrogen receptor binding assay

The LanthaScreen® TR-FRET Estrogen Receptor Alpha Competitive Binding Assay kit used for measuring estrogen receptor binding was purchased from Invitrogen. The assay was performed as described in the instruction manual. Incubation time was 2 h. Each data point was measured with four replicates. The TR-FRET assay was performed on a BMG LABTECH PHERAstar with a 337 nm excitation filter and 490 nm and 520 nm emission filters. The delay time was 100  $\mu\text{s}$  and the integration time was 200  $\mu\text{s}$ . Curves were fit using a sigmoidal dose-response equation in GraphPad™ Prism® 5.0.

## 4.4. Cell culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC number: HTB 22). Cells were cultured in 25 cm<sup>2</sup> tissue culture polystyrene flasks (Corning) containing growth medium and maintained at 37 °C in a humidified, 5%  $\text{CO}_2$  atmosphere. Complete growth medium was composed of Dulbecco's modification of eagles medium (DMEM) (Cellgro), 10% (v/v) fetal bovine serum (FBS) (Sigma–Aldrich), 2 mM L-glutamine (VWR), and antibiotics (100  $\mu\text{g}/\text{mL}$  penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin) (Sigma–Aldrich).

## 4.5. Cytotoxicity assays

The cytotoxicity of the compounds was determined using a colorimetric assay with an established dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is

metabolized in the mitochondria of viable cells to a formazan precipitate which is subsequently dissolved in 100% DMSO and the intensity of absorbance measured at 540 nm. The intensity of absorption is directly proportional to the number of viable cells. For these studies, MCF-7 cells were plated in a 96-well tray at a density of 5000 cells per well. On day 2 post plating the cells were first treated with the inhibitors netropsin (100  $\mu$ M) or fulvestrant (250  $\mu$ M) if needed, for 20 min, and then treated with the desired concentration of compounds. After an overnight exposure (16 h) to the respective compounds, the medium from each well was replaced with serum free DMEM supplemented with MTT (1 mg/mL). The cells were then incubated for 4 h at 37 °C/5% CO<sub>2</sub>. The resulting formazan precipitate was solubilized in DMSO. The absorbance was then measured at 540 nm using a Vmax plate reader from Molecular Devices. The experiment was done in replicates of 4 wells per concentration point and the average absorbance was plotted as a function of a percentage of the control cells that were only treated with an equivalent volume of DMSO.

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### Supplementary data

Supplementary data (includes a representative HPLC chromatogram showing separation of DNA adducts and <sup>1</sup>H and <sup>13</sup>C of all new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.026. These data include MOL files and InChIKeys of the most important compounds described in this article.

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