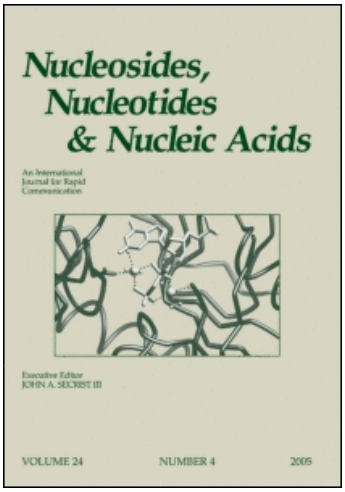

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
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To cite this Article Hadj-Bouazza, Amel , Teste, Karine , Colombeau, Ludovic , Chaleix, Vincent , Zerrouki, Rachida , Kraemer, Michel and Catherine, Odile Sainte(2008) 'Synthesis and Biological Activity of Mustard Derivatives of Thymine', Nucleosides, Nucleotides and Nucleic Acids, 27: 5, 439 — 448

To link to this Article: DOI: 10.1080/15257770802086872

URL: http://dx.doi.org/10.1080/15257770802086872

Nucleosides, Nucleotides and Nucleic Acids, 27:439–448, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802086872

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF MUSTARD DERIVATIVES OF THYMINE

Amel Hadj-Bouazza,[1] Karine Teste,[1] Ludovic Colombeau,[1] Vincent Chaleix,[1] Rachida Zerrouki,[1] Michel Kraemer,[2] and Odile Sainte Catherine[2]

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□ *The synthesis and biological activity of a novel DNA cross-linking antitumor agent is presented. The new alkylating agent significantly inhibited cell proliferation, migration and invasion as tested in vitro on the A431 vulvar epidermal carcinoma cell line.*

Keywords Nitrogen mustards; DNA-cross-linking; anticancer; thymine; microwave

INTRODUCTION

The nitrogen mustards chlorambucil, melphalan, cyclophosphamide, and ifosfamide are still among the most useful clinical agents for the treatment of a number of cancers.[1] All of them are bifunctional alkylating molecules which can react with *N*-7 of two different guanines in the major groove of a DNA molecule after the formation of aziridinium ions.[2,3] Provided that these guanines belong to different strands, these two reactions result in a cross-link which prevents uncoiling of the double helix. If the two guanines lie in the same strand, the result is called "limpet attachment." Monoalkylating agents can react only with one guanine *N*-7. Limpet attachment and monoalkylation do not prevent the opening of the double helix but do prevent vital DNA processing enzymes from accessing DNA. The

Received 17 September 2007; accepted 22 February 2008.
Financial support from the "Conseil Régional du Limousin" is gratefully acknowledged. The authors are grateful to Dr. Michel Guilloton for useful comments on the manuscript.
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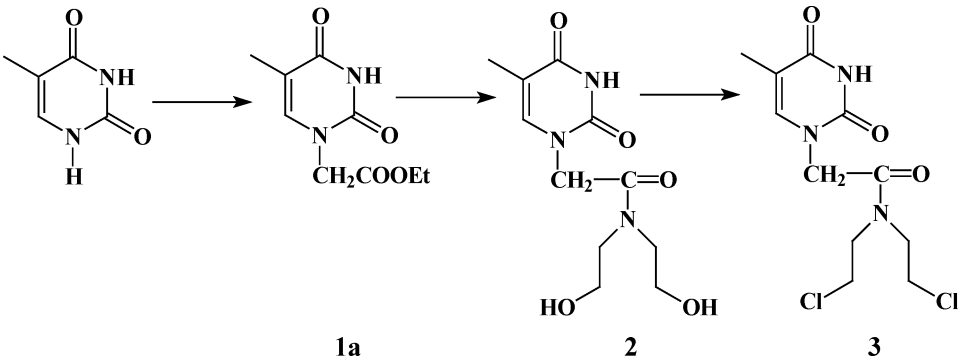


SCHEME 1 Strategy of synthesis.

outcomes of all these alkylation reactions are an inhibition of cell division or a triggering of apoptosis. Since cancer cells generally divide more rapidly than do healthy ones, the former prove more sensitive to DNA damage and, hence, the use of alkylating agents to treat a variety of tumors.

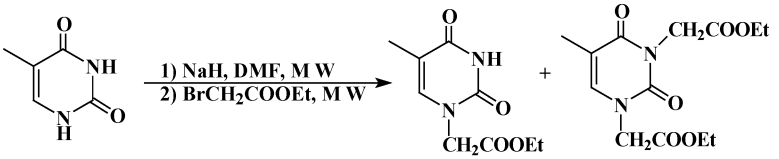
In connection with our research program on modified nucleosides designed for cancer therapy, we report in this article the efficient synthesis of a new dialkylating agent comprised of a thymine bearing a bis(β-chloroethyl)amine moiety.

RESULTS AND DISCUSSION

The general procedure for the synthesis of this modified nucleoside (displayed in Scheme 1) consists of three steps.

The first step consists in the regioselective alkylation of thymine. Two methods have been reported for the preparation of *N*-1 substituted thymine. The first one requires four steps including a protection/deprotection sequence: thymine reacts with a benzoylating[4] or silylating[5] agent to give the corresponding *N*-1,*N*-3-bisprotected derivative. This compound is then converted into *N*-3 benzoyl or silyl thymine and, after substitution, the product is deprotected to give *N*-1 alkylated thymine.

Another option is the direct alkylation of thymine, since the standard conditions described in the literature,[6] do not lead to regioselective substitution and, in addition to *N*-1 alkylation, *N*-1,*N*-3 bis-alkylation also is observed. These results strongly suggest that the slight difference in reactivity between *N*-1 and *N*-3 is the cause of relatively poor yields of the mono *N*-1 alkylation reaction. Due to our interest in finding convenient synthesis of monoalkylated thymine, we reinvestigated this reaction. We propose in the present work the use of microwave irradiation for activation of thymine alkylation and avoidance of the subsequent *N*-3 alkylation. Microwave heating presents several advantages, such as a remarkable reduc-



1a **1b**

SCHEME 2 Alkylation of thymine.

tion in reaction times and, in some cases, cleaner reactions and a higher regioselectivity.[7]

In a typical procedure, a solution of thymine with sodium hydride in dried DMF was irradiated for 3 minutes, then alkyl bromide was added and the mixture was irradiated again for 3 minutes (Scheme 2).

Further, alkylating reaction in the presence of various amounts of NaH and alkyl bromide has been studied. Different conditions of temperature and power of activation also were tested. Selected results are summarized in Table 1.

Data reported in Table 1 showed that an optimal result (84%) was obtained after 3 minutes of activation (120°C, 300 W) in the presence of 1.1 equiv. of NaH and 1.1 equiv. of alkyl bromide. *N*-1 Monoalkylated thymine was obtained in 74% yield together with a small amount of *N*-1,*N*-3-bisalkylated derivative (10%).

By refluxing **1a** in dry EtOH with an excess of diethanolamine, compound **2** was obtained in 93% yield after purification. Chlorination of compound **2** was achieved with thionyl chloride. The use of DMF as a solvent resulted in a poor yield (10%) even in the presence of a large excess of thionyl chloride. A better yield of compound **3** was obtained in CH_2Cl_2 (35%), but the yield was optimized (66%) when chlorination was realized in presence of an excess of thionyl chloride without any solvent (Scheme 1).

TABLE 1 Selected results of thymine alkylation

Entry	NaH (eq.)	$BrCH_2COOEt$ (eq.)	Activation	Yield[a] (%)	Selectivity[b] 1a/1b
1	1.1	1.1	60°C, 100 W	37	54/46
2	1.1	1.5	60°C, 100 W	42	40/60
3	1.1	2.3	60°C, 100 W	45	33/67
4	2	2.3	60°C, 100 W	50	30/70
5	1.1	1.1	100°C, 200 W	49	57/43
6	1.1	1.1	100°C, 250 W	54	69/31
7	1.1	1.1	100°C, 300 W	66	80/20
8	1.1	1.1	120°C, 300 W	84	88/12

[a]Global yields (compound 1a[7] plus compound 1b[7]).
[b]Based on individual yields for compounds 1a and 1b.

The structures were established by NMR and mass spectra. The mass spectrum of **3** showed the presence of the protonated species $(M+H)^+$ m/z 308, m/z 310 and m/z 312 consequence of the two chlorine isotopes and in addition $(M+NH_4)^+$ m/z 325, m/z 327, and m/z 329.

BIOLOGICAL EVALUATION

First, we explored in vitro if *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) could inhibit the A431 cell proliferation. A431 human squamous carcinoma cells represent a good model of an aggressive, highly angiogenic and metastatic tumor.[8] A431 cells display an increase of epidermal growth factor receptors (EGFR) and produce large amounts of vascular endothelial growth factor (VEGF)[9] promoting neovascularization and angiogenesis.[10] Increased EGFR expression renders A431 cells less dependent upon an exogenous source of epidermal growth factor (EGF) and enhances the EGF-induced mitogenic responses of squamous cell carcinoma cell lines compared with human epidermal keratinocytes, contributing to the invasiveness of malignant cells.[11]

In Vitro Effects of *N,N*-Di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (3) on Proliferation of A431 Tumor Cell Line

To investigate the effects of compound **3** on the cell proliferation, the tumor cells were treated with increasing doses of compound **3** ranging from 15 μM to 1 mM (Figure 1).

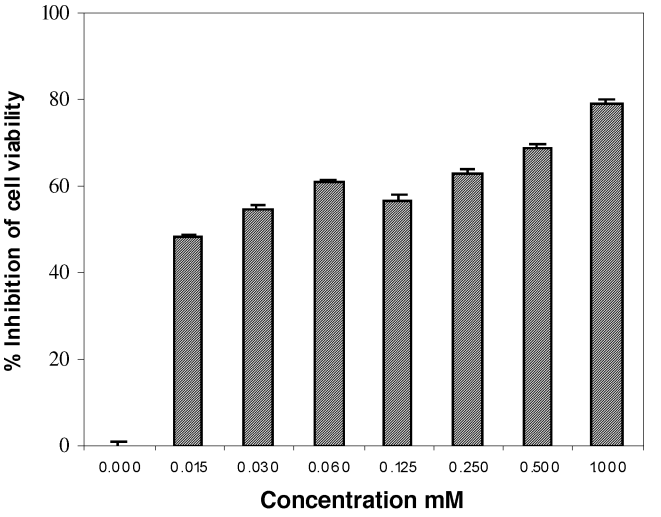


FIGURE 1 Dose-dependent effects of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) on A431 cell viability. A431 cells were treated with increasing concentrations (15 μM to 1 mM) of compound **3** for 72 hours. Results are mean ± SEM of three independent experiments.

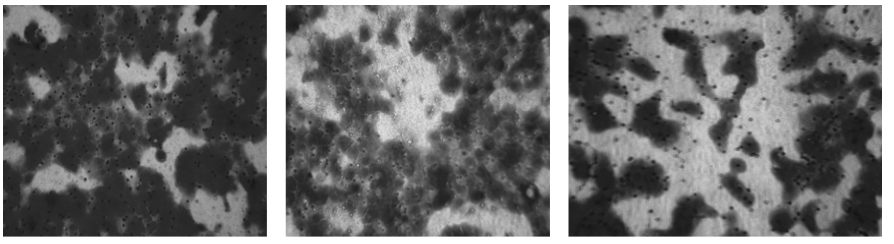


FIGURE 2 Effects of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) on the migration of A431 cells seeded on a fibronectin matrix in the upper chamber, with 10% FCS added to the lower chamber. Fewer cells migrated to the lower chamber in the presence of compound **3**. Migration of A431 cells was inhibited by 60% and 68% by compound **3** at 50 μM and 100 μM, respectively. Original magnification $\times$ 200.

Compound **3** inhibited tumor cell proliferation in a dose-dependent manner. The concentration inducing 50% of maximal inhibition (IC_{50}) was 60 μM.

Then, as most tumors cells are able to disseminate via vascular or lymphatic vessels,[12] the effects of compound **3** on tumor cell migration and invasion has been assessted. Invasion of tumor cells in the extracellular matrix is assumed by proteases such as metalloproteases. Finally the effects of **3** on the expression of metalloproteases was studied.

Cell Migration Assay

In the presence of a chemotatic stimulus such as foetal calf serum (FCS) in the lower part of the Boyden migration chamber, A431 cells migrated through the pores to the lower surface of the membrane. *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) significantly reduced cell migration. In comparison to the untreated control cells, migration of A431 significantly decreased 60% ($p<0.05$) and 68% ($p<0.05$) in presence of 50 μM and 100 μM of compound **3**, respectively (Figure 2).

Cell Invasion Assay

A Matrigel invasion assay was performed to study the effect of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) (50 μM and 100 μM) on the invasive ability of A431 cells. Compared with untreated control cells, invasion of A431 significantly dropped by 63% ($p < 0.05$) and 80% ($p < 0.05$) in presence of 50 μM and 100 μM of **3**, respectively (Figure 3).

N,N-Di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (3) Reduces Expression Metallo-Proteases

Cell migration that takes place during angiogenesis requires a degradation of the extracellular matrix by proteases such as matrix metalloproteases

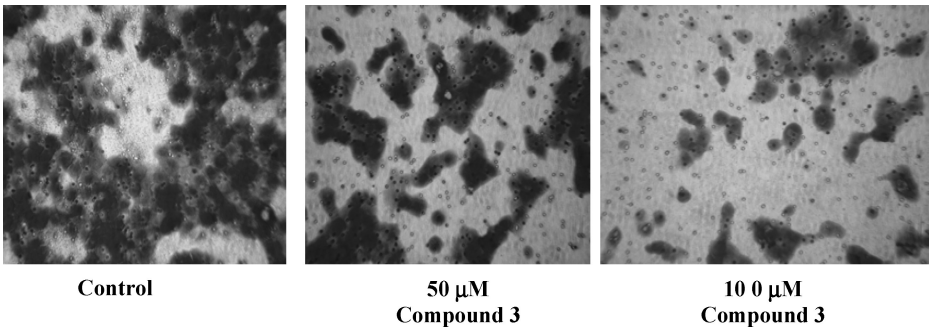


FIGURE 3 Effects of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) on the invasion of A 431 cells seeded on a Matrigel matrix in the upper chamber, with 10% FCS added to the lower chamber. Fewer cells invaded the lower chamber in the presence of compound **3**. Invasion of A431 cells was inhibited by 63% and 80% by compound **3** at 50 μM and 100 μM respectively. Original magnification × 200.

(MMP).[13] Since compound **3** inhibited migration and invasion of A431 cells, compound **3** was examined by zymography to investigate whether this compound could affect the secretion of MMP9 and MMP2 gelatinases by A431 cells. Amounts of ProMMP9, MMP9 and ProMMP2 secreted in the medium after 72 hours of treatment with 4μM or 16 μM of compound **3** were analyzed by quantitative zymography and normalized to cell number. Expressions of MMP9, ProMMP9, and ProMMP2 decreased by 85, 50, and 25%, respectively, as compared to control cells and whichever concentration of compound **3**, 4μM or 16 μM (Figure 4).

The antimigrative and antiproliferative effects of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide can therefore been explained, as least in part, by an inhibition of MMP9 expression.

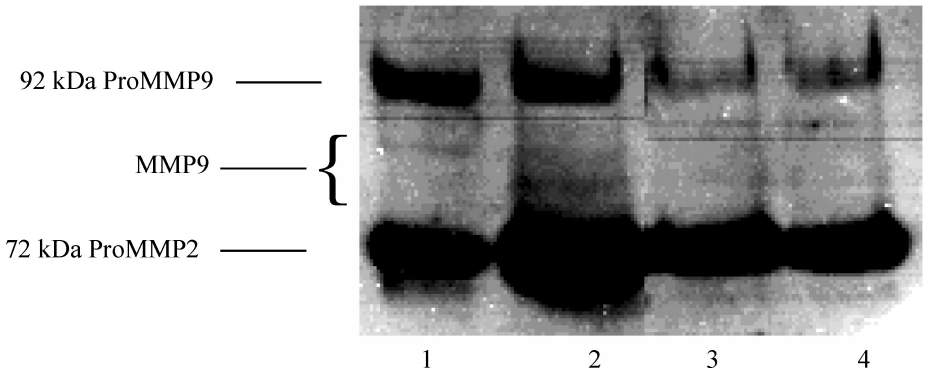


FIGURE 4 Effects of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide (**3**) on ProMMP9, MMP9 and ProMMP2 expression by A431 cells. Conditioned media were collected after 72 hours of incubation, normalized to cell number, and subjected to gelatin zymography. Lane 1: conditioned medium from a positive control (HT 1080 cells); lane 2: untreated A431 cells, lanes 3 and 4: A431 cells treated with compound **3** at 16 and 4 μM, respectively.

CONCLUSION

In the present work, we report the synthesis of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl) acetamide and preliminary results of a biological evaluation on the highly invasive A431 tumor cell line suggests that *N,N*-di-(2-chloro-ethyl)-2-(thymin-1-yl)acetamide could be efficient in inhibiting tumor growth and metastasis. Further investigation to demonstrates that this compound can act as an anticancer drug are actually in progress in our laboratory and additional data will be published elsewhere.

EXPERIMENTAL

Chemistry

General. Microwave irradiation were performed with a monomode reactor (MicroSYNTH from Milestone) with focused waves. Thin−layer chromatography (TLC) was performed on Merck aluminum foils precoated with silica gel 60 F_{254}, with detection by UV. Silica gel (Merck Kieselgel 60, 15–40 μm) was used for flash chromatography. ^{1}H NMR spectra were recorded using a Brucker DPX-400 spectrometer with tetramethylsilane as internal standard. The chemical shifts are given in δ and coupling constants expressed in Hz.

Alkylation of Thymine. Thymine (756 mg, 6.0 mmol) was stirred with sodium hydride (1.1 eq.) in DMF (20 mL) under microwave irradiation over 3 minutes (120°C, 300 W). Ethyl bromo-acetate (0.75 mL, 1.1 eq.) was then added and the reaction mixture was irradiated under stirring for 3 minutes at 120°C (300 W). After evaporation and purification by chromatography with an elution gradient of petroleum ether/acetone, *N*-1 alkylthymine (white solid) and *N*-1,*N*-3 bisalkylated thymine (oil) were recovered in 74% (1g) and 10% (178 mg) yields, respectively.

1-Ethoxycarbonylmethylthymine. M.p. = 166°C, IR (ν_{max}, cm^{-1}) : 3100 (N-H), 1720, 1700 (C = O), 1670 (C = C). ^{1}H NMR (DMSO-d_6, δ) : 11.30 (s, 1H, N-H), 7.48 (br s., 1H, H-6), 1.76 (br s., 3H, CH_3-thym), 4.45 (s, 2H, CH_2), 4.14 (q, 2H, J = 7.1 Hz, $C\underline{H}_2CH_3$), 1.21 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$). ^{13}C NMR (DMSO-d_6, δ) : 168.1 ($\underline{C}O$), 164.2 (C-4), 150.8 (C-2), 141.4 (C-6), 108.4 (C-5), 61.0 ($O\underline{C}H_2CH_3$), 48.3 ($\underline{C}H_2$), 13.9 ($\underline{C}H_3$ thym), 11.7 ($OCH_2\underline{C}H_3$).

1,3-Di-(1-ethoxycarbonylmethyl)Thymine. IR (ν_{max}, cm^{-1}) : 1721, 1706 (C = O),1675 (C = C). ^{1}H NMR (DMSO-d_6, δ) : 7.50 (q, 1H, J = 1.0 Hz, H-6) ; 6.11 (q, 2H, J = 7.1 Hz, $C\underline{H}_2CH_3$) ; 5.9 (q, 2H, J = 7.1 Hz, $C\underline{H}_2CH_3$) ; 4.65 (s, 2H, CH_2) ; 4.47 (s, 2H, $\underline{C}H_2$), 1.77 (d, 3H, J = 1 Hz, CH_3-thym) ; 1.27 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$); 1.23 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$).

N,N-Di-(2-hydroxyethyl)-2-(thymin-1-yl)acetamide. 1-Ethoxycarbonyl-methylthymine (1g, 4.72 mmol) was stirred with diethanolamine (4.50 mL,

47.20 mmol) in EtOH (30 mL) under reflux overnight. The mixture was evaporated and then purified by chromatography with an elution gradient of CH_2Cl_2/EtOH. Compound **2** was obtained in 93% yield (1.18 g).

M.p. = 193°C. ^{1}H NMR (CD_3OD, δ) : 7.17 (q, 1H, J = 1.0 Hz, H-6), 1.91 (d, 3H, J = 1.0 Hz, CH_3), 4.73 (s, 2H, CH_2), 3.79 (t, 2H, J = 5.2 Hz, (OCH_2CH_2N), 3.74 (t, 2H, J = 5.5 Hz, OCH_2CH_2N), 3.57 (t, 2H, J = 5.2 Hz, OCH_2CH_2N), 3.53 (t, 2H, J = 5.5 Hz, OCH_2CH_2N). ^{13}C NMR (CD_3OD, δ) : 168.8 (CON), 166.1 (C-4), 152.4 (C-2), 142.7 (C-6), 110.8 (C-5), 60.1 (OCH_2CH_2N), 59.9 (OCH_2CH_2N), 51.4 (OCH_2CH_2N), 50.1 (OCH_2CH_2N), 49.3 (CH_2), 12.2 (CH_3 thym). MS (IS) m/z = 272 ($M+H^+$), 289 ($M+NH_4^+$).

N,N-Di-(2-chloro-ethyl)-2-(thymin-1-yl)acetamide. A solution of **2** (100 mg, 0.37 mmol) in thionyl chloride (2 mL) was stirred for 5 hours at room temperature. After evaporation, the crude product was subjected to preparative TLC using AcOEt/EtOH, 90/10, yielding **3** (75 mg, 66%) as a white solid.

M.p. = 188°C. ^{1}H NMR (CD_3OD, δ) : 7.02 (s, 1H, H-6), 1.93 (s, 3H, CH_3), 4.62 (s, 2H, CH_2), 3.85 (t, 2H, J = 5.8 Hz, ($ClCH_2CH_2N$), 3.77 (t, 2H, J = 5.8 Hz, $ClCH_2CH_2N$), 3.71 (m, 4H, $ClCH_2CH_2N$), ^{13}C NMR (CD_3OD, δ) : 167.4 (CON), 164.5 (C-4), 151.2 (C-2), 141.1 (C-6), 110.9 (C-5), 50.3 ($ClCH_2CH_2N$), 49.2 ($ClCH_2CH_2N$), 41.5 ($ClCH_2CH_2N$), 41.2 ($ClCH_2CH_2N$), 48.2 (CH_2), 12.3 (CH_3 thym). MS (IS) m/z = 308, 310 and 312 ($M+H^+$), m/z = 325, 327 and 329 ($M+NH_4^+$).

Biological Tests

A431 Cell Culture. A431 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U.mL^{-1} streptomycin (all obtained from Life Technologies, Inc., USA), at 37°C in a 5% CO_2 humidified atmosphere.

Cell Viability Experiments. Cell viability was evaluated using the MTT microculture tetrazolium assay,[14] which is based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) into purple formazan crystals. Cells were seeded at a density of 5×10^3 cells in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 hours. Then, the medium was removed and replaced by 2% FCS-medium containing increasing concentrations of *N,N*-di-(2-chloro-ethyl)-2-(thymin-1-yl)acetamide varying from 1 mM to 15 μM. After 72 hours incubation, the cells were washed with phosphate buffered saline (PBS, Life Technologies) and incubated with 0.1 mL MTT (2 mg. mL^{-1}, Sigma-Aldrich, USA) for an additional 4 hours at 37°C. The insoluble product was then dissolved by addition of 200 μL DMSO (Sigma-Aldrich). Absorbances

corresponding to solubilized formazan pellets (which reflect the relative viable cell numbers) was measured at 570 nm using a Labsystems Multiskan MS microplate reader.

Concentration-response curves were constructed and IC_{50} values (concentration of the compound inhibiting 50% of cell proliferation) were determined.

Cell Migration Assay. The influence of *N,N*-di-(2-chloro-ethyl)-2-(thymin-1-yl)acetamide on migration of A431 cells was investigated as described previously[14] using Boyden invasion chambers with 8 μm pore size filters coated with 100 μL of fibronectin (100 μg.mL^{-1}, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were allowed to stand overnight at 4°C. 5×10^4 untreated or 24 hours *N,N*-di-(2-chloro-ethyl)-2-(thymin-1-yl)acetamide (at 100 μM or 50 μM)-pretreated A431 cells were added to each insert (upper chamber). A strong chemoattractant for A431 cells (10% FCS) was added to the lower chamber. After 24 hours incubation at 37°C in a 5%CO_2−incubator, nonmigrated cells were removed by scraping and migrated cells were fixed in MeOH and stained with haematoxylin. Cells migrating on the lower surface of the filter were counted in 10 fields using a Zeiss microscope. Results were expressed as a percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

Cell Invasion Assay. Cell invasion experiments were performed with Boyden chambers as described above. The inserts were coated with Matrigel membrane matrix (Falcon, Becton Dickinson Labware, Bedford, MA, USA). A431 cells (5×10^4) were seeded in the upper well of the Boyden chamber and 10% FCS was added to the lower chamber; *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide was added at 100 μM or 50 μM. After 24 hours at 37°C in a 5%CO_2−incubator, noninvaded cells in the upper chamber were wiped with a cotton swab and the filters were fixed, stained, and counted. Results were expressed as a percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

Zymography. A431 cells were seeded at a density of 5×10^5/well into 6-well tissue culture plates in DMEM-10% FCS. Cells were allowed to adhere for 24 hours and then incubated with 4 or 16 μM of *N,N*-di-(2-chloroethyl)-2-(thymin-1-yl)acetamide. Conditioned media were collected 72 hours after treatment, normalized to cell number, mixed with non reducing Laemmli sample buffer, and subjected to 10% SDS-PAGE containing 0.1% (w/v) gelatin. The gel was washed 3 times at room temperature in a solution containing 2.5% (v/v) Triton X-100 in H_2O and incubated at 37°C for 24 hours in 50 mM Tris/HCL, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.05% Brij 35. The gel was stained for 60 minutes with 0.5% (w/v) R-250 Coomassie blue in 30% MeOH (v/v)/10% AcOH (v/v). ProMMP9, MMP9 and ProMMP2 were visualized as white zones on the gels indicating the gelatinolytic activity of proteinases. Gelatinase activity was quantified using a NIH image program.

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