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Report

The effect of DNA-alkylating agents on gene expression from two integrated reporter genes in a mouse mammary tumor line

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A model system was developed to investigate the effects of DNA alkylating agents on cellular gene expression. The cytomegalovirus immediate-early promoter (CMV) and the mouse mammary tumor virus promoter (MMTV) were coupled separately to the luciferase reporter gene and stably expressed in cultured cells. The change in luciferase activity was used as a measure of gene expression inhibition. Seven well-characterized DNA alkylating agents of varied DNA adduct-forming ability were evaluated in this system. The major groove binders/intercalators (that form guanine adducts) increased CMV–luciferase activity above background, while minor groove binders (that form adenine adducts) all decreased it. The MMTV–luciferase activity was remarkably different to the CMV–luciferase activity and was inhibited to the greatest extent by the minor groove alkylators. One of these, a polybenzamide with spatially separated alkylating groups, inhibited gene expression to a greater extent than inhibition of general DNA or RNA synthesis. [© 2002 Lippincott Williams & Wilkins.]

Key words: DNA alkylating agent, gene expression, reporter gene.

Introduction

Compounds that covalently react with double-stranded DNA to form adducts are an important class of anticancer agents. They were among the earliest anticancer drugs, and although falling out of favor because of their relative lack of selectivity, low therapeutic index and substantial mutagenic/carcinogenic effects,[1] are still used, and today are enjoying renewed research interest in a variety of prodrug forms.[2] The nature of the DNA adducts formed by these compounds has been well explored and shown to vary widely. While it is assumed that the adducts induce a lethal cellular response, the mechanisms by which they do so are less well defined and also vary. DNA adducts have been shown to inhibit a range of biochemical functions, including DNA replication, transcription and the binding of transcription factors (reviewed in Zewail-Foote and Hurlay[3]). As cancer is a product of aberrant gene expression, the apparent ability of these simple compounds to modulate gene expression in a selective way suggests a new design potential for alkylating agents.

Even the simplest DNA alkylators such, as the clinical drugs cisplatin (**1**) and chlorambucil (**2**), exhibit some degree of both sequence- and regiospecificity of alkylation, favoring certain DNA sites. Cisplatin (**1**) reacts primarily at G-N^7 DNA sites in the major groove, and forms primarily G-N^7/G-N^7 intrastrand cross-links, while chlorambucil (**2**) reacts at both G-N^7 and A-N^7 sites,[4] although forming primarily G-N^7/G-N^7 interstrand cross-links.[5] Carrying groups can modify these patterns of alkylation. Thus the intercalator-linked platinum analog (**3**) alkylates DNA more rapidly than cisplatin and shows some difference in sequence specificity, away from alkylation at runs of consecutive guanines towards damage at many more G sites.[6] The intercalator-linked aniline mustard (**4**) also shows some differences to chlorambucil, but these are towards more exclusive alkylation at isolated G sites.[7] In contrast, aniline mustards linked to the minor groove binding bisbenzimidazole (compound **5**) alkylates primarily at A sites,[8] while the polybenzamide (compound **6**) shows even more dramatic changes, switching to alkylation almost exclusively at

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A-N^7 sites, particularly in runs of As.[4] Finally, the CBI compound (**7**) is an example of an intrinsically selective alkylator, reacting at A-N^3 sites in poly(A) sequences.[9]

This sequence selectivity comes from a combination of the heterogeneous structure and chemical reactivity of DNA, and from the molecular recognition properties and reaction mechanism of the alkylators. This knowledge has led to an increased focus on developing sequence selective agents, with the aims of improving their potency and their specificity towards particular DNA sites, in the hope that this will translate also into higher selectivity towards tumor cells. Various methods have been used to design in selectivity, including the use of chemically regiospecific alkylating units such as CBI (A-N^3 specific) (e.g. compound **7**)[9,10] and PBDs (G-$2NH_2$ specific),[11] and the use of sequence-specific 'carriers'.[12] Both intercalating (e.g. acridines, as in compounds **3** and **4**) and minor groove-binding moieties (e.g. polybenzamides as in compound **5** and bisbenzimidazoles as in compound **6**) have been used as carriers, with minor groove binders showing the greatest promise for sequence selectivity. This has culminated in the recent development of synthetic polyamides that can recognize, and specifically deliver alkylating agents to, selected bases in pre-defined DNA sequences.[13] These compounds have been shown to inhibit transcription factor binding, the action of RNA polymerase II and viral replication in human cells.[14]

Techniques developed to study the effects of DNA adducts on the cellular machinery include *in vitro* reconstituted assays with purified DNA templates, and simple bacterial promoters and transcription factors.[15] Transient cell transfection with reporter genes, followed by treatment with DNA alkylators, has also been used.[16] More detailed analyses have investigated the ability of certain DNA binders to inhibit transcription factor binding with mobility shift assays.[17]

We have developed an *in vivo* model system to investigate the effects of DNA alkylating agents on gene expression. Two viral promoters [cytomegalovirus immediate-early promoter (CMV) and the mouse mammary tumor virus promoter (MMTV)] were coupled with the luciferase reporter gene and stably expressed in cultured cells. Cells were treated with seven DNA alkylating agents of varied and well-characterized chemistry and DNA adduct-forming ability (compounds **1–7**, Figure 1) to investigate their effects on gene expression. This system was developed as a rapid and simple screen to characterize the ability of DNA alkylators to modulate gene expression. From the panel of seven alkylators we have identified one, the polybenzamide (**5**), which inhibits gene expression from both promoters to a greater extent than DNA or RNA synthesis alone.

Methods

Cell culture

The cell lines used in this study were the mouse mammary tumor line EMT6, and the transfected derivatives EG109 and EG112. Cells were grown in α-minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (Life Technologies, Gibco, Gaithersburg, MD), and 100 U/ml of penicillin and 100 μg/ml of streptomycin. They were passaged in 25-cm^2 tissue culture flasks in an atmosphere of 5% CO_2 in air and were subcultured weekly, using 0.1% trypsin (Difco, Franklin Lakes, NJ) in citrate saline (trisodium citrate dihydrate, 4.4 g/l, KCl, 10 g/l; pH 7.3).

Measurement of DNA and RNA synthesis

EMT6 cells were plated in 96-well plates at 1000 cells/well and allowed to grow overnight before drug addition. Compounds were added to the plates in quadruplicate and incubated for 2 h before the medium was replaced with fresh culture medium. Cells were incubated for either 4 or 24 h before DNA or RNA synthesis was measured. For the measurement of DNA synthesis, [^{3}H]thymidine (20 Ci/mmol; 0.04 μCi/well), thymidine (0.1 μM/well) and 5-fluoro-2′-deoxyuridine (0.1 μM/well) was added 2 h before culture harvest. For the measurement of RNA synthesis [5-^{3}H]uridine (25 Ci/mmol; 0.04 μCi/well) was added 4 h before culture harvest. Cells were released from the 96-well plate by the addition of pronase (1 mg/ml) (Sigma, St Louis, MO; no. P-8811) dissolved in 4 mM EDTA/PBS and incubated for 30 min at 37°C. Cells were harvested from the 96-well plates using a Wallac (Boston, MA) cell harvester onto glass fiber filter mats. After drying at 37°C, 10 ml of Betaplate Scint (Wallac) was added and the filter mats counted using a Wallac 1205 Betaplate scintillation counter. All data analysis was carried out using Microsoft Excel.

Plasmid construction

Two plasmids containing the luciferase reporter gene were constructed using standard molecular biology

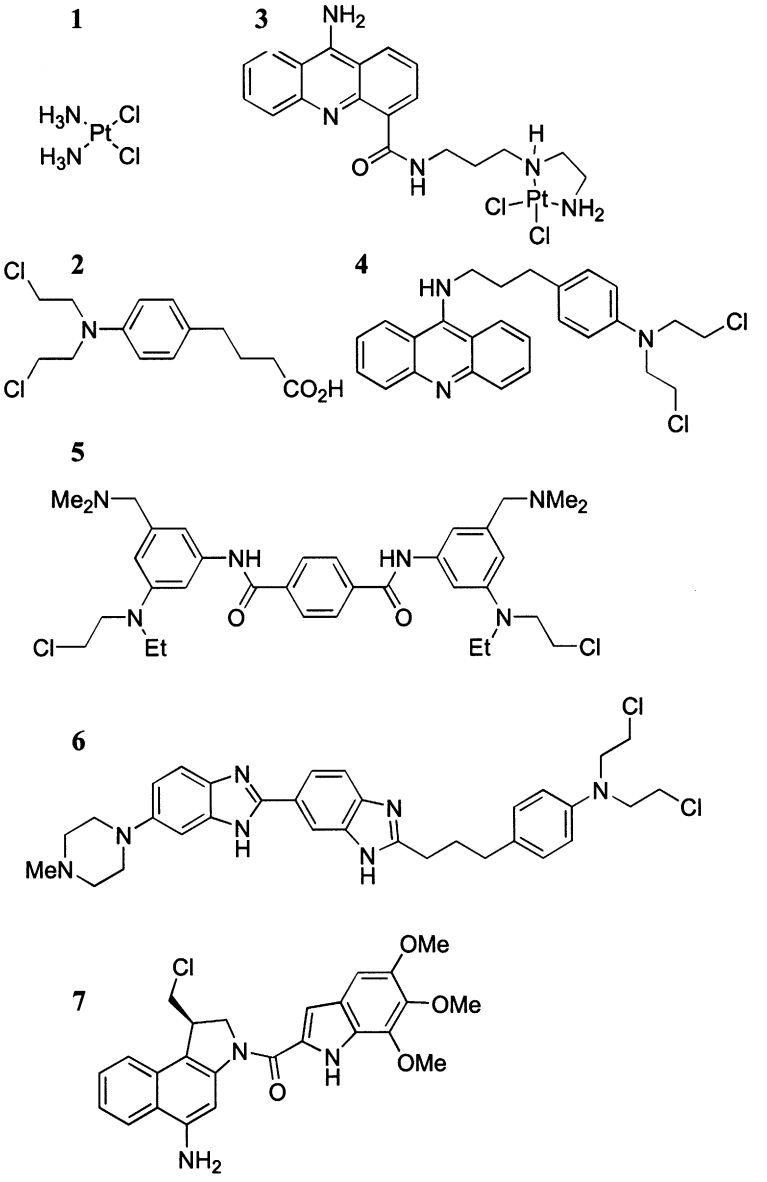

Figure 1. Structures of the alkylators studied.

cloning techniques.[18] All enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN) unless otherwise stated. Plasmids were purified using QIAgen (Valencia, CA) plasmid purification kits. The plasmid pMG109 contains the luciferase open reading frame 3′ to the CMV promoter. The plasmid pMG112 contains the luciferase open reading frame 3′ to the MMTV long terminal repeat. For construction of pMG109 the luciferase open reading frame was excised from the plasmid pGL3 (Promega, Madison, WI) using the restriction enzymes *Hin*dIII and *Xba*I. The resulting 1.7-kb fragment was ligated directly into the mammalian expression vector pcDNA3 (5.4 kb; Invitrogen, Carlsbad, CA) cut with *Hin*dIII and *Xba*I, resulting in the 7.1 kb vector pMG109. For construction of the plasmid pMG112 the CMV promoter from plasmid pcDNA3 was first removed by digesting pcDNA3 with *Bgl*II. The resulting 5′ overhang was removed with Mung Bean nuclease (New England Biolabs, Beverly, MA). The linearized plasmid was then digested with *Hin*dIII to remove the CMV promoter and the resulting 5′ recessed end filled with Klenow enzyme. The two blunt termini were then ligated using T4 DNA ligase. The resulting 4.5-kb plasmid (pMG103) was digested with *Hin*dIII and *Xba*I, and ligated to the luciferase

open reading frame (1.7 kb) from pGL3. The new plasmid was named pMG110. The MMTV promoter was kindly provided to us by Jaquelin Dudley (Austin, TX) as the plasmid pLC-1. The MMTV promoter was excised by digestion with *Hin*dIII from pLC-1 and the resulting 1.4-kb fragment ligated with pMG110 digested with *Hin*dIII. The resulting plasmids were selected for correct orientation of the MMTV promoter. The correct 7.6-kb plasmid was named pMG112.

Cell line construction

The cell line EMT6 was transfected with either pMG109 or pMG112 to obtain stable transfected lines. EMT6 cells were plated into 60 cm culture dishes at 3.5×10^5 cells/dish and incubated for 24 h. Cells were then transfected with 2 μg of plasmid using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's instructions. The following day transfected cells were passaged into 10 cm culture dishes and stable integrates selected with 400 μg/ml G418 (Life Technologies). After 7 days, the surviving cells were plated at 5 cells/ml or 25 cells/ml into 96-well plates containing 400 μg/ml G418 to select for single clones. After a further 7 days, wells that contained cells were passaged into T25 culture flasks and luciferase expression analyzed. Clones that were selected for further work were grown in culture medium with a maintenance concentration of 200 μg/ml G418. The cell line developed with the CMV–luciferase construct was called EG109 and the line developed with the MMTV–luciferase construct was called EG112.

Luciferase expression analysis

The cell lines EG109 and EG112 were plated in duplicate at 5×10^4 cells/30-cm culture plate and incubated for 24 h before drug addition. Drug was added to each plate and after a 2-h incubation was replaced with fresh culture medium. For EG109, the cells were harvested 6 h after drug removal. For EG112, dexamethasone (1 μM final concentration; Sigma) was added immediately after drug removal and the cells harvested after a further 6 h. Cells were lysed by the addition of 400 μl of 1 $\times$ reporter lysis buffer (Promega) and the cells scraped off the plate using an inverted 200 μl pipette tip. The cell lysate was collected and underwent one freeze/thaw cycle, before centrifugation at 12 000 *g* for 5 min at 4°C. The pellet was discarded and the supernatant retained for luciferase assay as per the manufacturer's instructions (Promega). Luciferase activity was determined using a Wallac Trilux luminometer with 20 μl samples in duplicate. The protein concentration was assayed using the BCA protein assay. Cell extracts (50 μl) were incubated with 100 μl of BCA reagent (Sigma) for 30 min in a 96-well plate. Absorbance was measured on a dual wavelength spectrophotometer at 570 nm. Protein concentration was determined from a BSA standard curve dissolved in 1 $\times$ reporter lysis buffer. Luciferase activity was defined as units of luciferase/μg of protein.

Results

Inhibition of DNA and RNA synthesis by the alkylating agents

The ability of the compounds to inhibit DNA and RNA macromolecular synthesis was tested to determine the appropriate concentration to dose and measure their effects on gene expression. The parent cell line of the transfectants, EMT6, was exposed to the compounds for 2 h, and DNA and RNA synthesis measured a further 4 h after drug removal. All except one of the compounds inhibited both DNA and RNA synthesis in a dose-dependent manner, as measured by incorporation of [^{3}H]thymidine and [^{3}H]uridine (Table 1, and see Figures 3 and 4). Chlorambucil (**2**) did not inhibit [^{3}H]uridine incorporation beyond 80% of the control at this time point (although [^{3}H]thymidine was inhibited in a dose-dependent manner), but did so after 24 h (data not shown). Cisplatin, chlorambucil, and compounds **4**, **6** and **7** inhibited DNA synthesis to a greater extent than RNA synthesis, while compound **5** inhibited both equally, and the platinum analog **3** inhibited RNA slightly more than DNA synthesis. The CBI minor groove binder **7** was the most potent inhibitor of DNA and RNA synthesis, with IC_{50}s in the low nM range (Table 1). The minor groove targeted mustards **4**–**6** were the next most potent with IC_{50}s in the high nM range, while chlorambucil and the platinum analogs **1** and **3** were the least potent with IC_{50}s in the μM range.

Time course of luciferase activity

Luciferase expression from the two cell lines (EG109 and EG112) was followed over a 24-h period to assess the dynamics of luciferase production and

Table 1. Inhibitory effects of DNA alkylators on DNA synthesis, RNA synthesis and luciferase activity

Compound	[^{3}H]Thymidine incorporation (μM)	[^{3}H]Uridine incorporation (μM)	MMTV–luciferase activity (μM)	CMV–luciferase activity (μM)
1	19	35	40	>100
2	94	>100	>100	>50
3	3.1	2.0	5.2	>10
4	0.33	0.59	1.0	>5
5	0.56	0.39	0.24	>2
6	0.91	1.4	1.2	>2
7	0.003	0.023	>0.025	>0.025

IC_{50} values were calculated using the program Calcusyn (Biosoft) and the median-effect principle of Chou and Talalay.[26] For each alkylator, all data points less than 100% were used in the calculation.

determine the most appropriate time for assaying following drug treatment. Cells were passaged from cultures in log phase into fresh culture dishes and the luciferase activity measured at 0, 2, 4, 8 and 24 h (Figure 2A and B). For EG112, the glucocorticoid dexamethasone (1 μM) was added at 2 h. Both cell lines were exposed to 10 and 50 μM cisplatin for the first 2 h of the time course to evaluate the effect the alkylating agent would have on gene expression and hence luciferase activity.

The cell line EG109 (CMV–luciferase construct) showed continuous luciferase activity over the 24-h period measured, although a peak in activity was detected 8 h after passaging (Figure 2A). This cell line contains the luciferase gene under control of the CMV immediate early promoter. This promoter was assumed to be constitutive, but it has recently been shown that it is stress induced in a p38-dependent manner through the SAPK pathway.[19] Environmental stresses such as heat shock, osmotic shock and cytotoxic drugs have been demonstrated to induce this promoter. It is thus unsurprising that after passaging of the cells there is a peak in luciferase activity. For time points after 24 h the luciferase activity slowly declined (data not shown). Treatment with 10 μM cisplatin had no effect on luciferase activity at the earlier time points, although an increase compared to the control was observed at 24 h (Figure 2A). Treatment with 50 μM cisplatin increased luciferase activity at all time points after 2 h.

The cell line EG112 (MMTV–luciferase construct) had no detectable background luciferase activity at the beginning of the time course (Figure 2B). This cell line contains the luciferase gene under control of the inducible MMTV promoter and luciferase activity was only detected following addition of the glucocorticoid dexamethasone. A peak in luciferase activity was observed at 8 h, which is 6 h after induction of

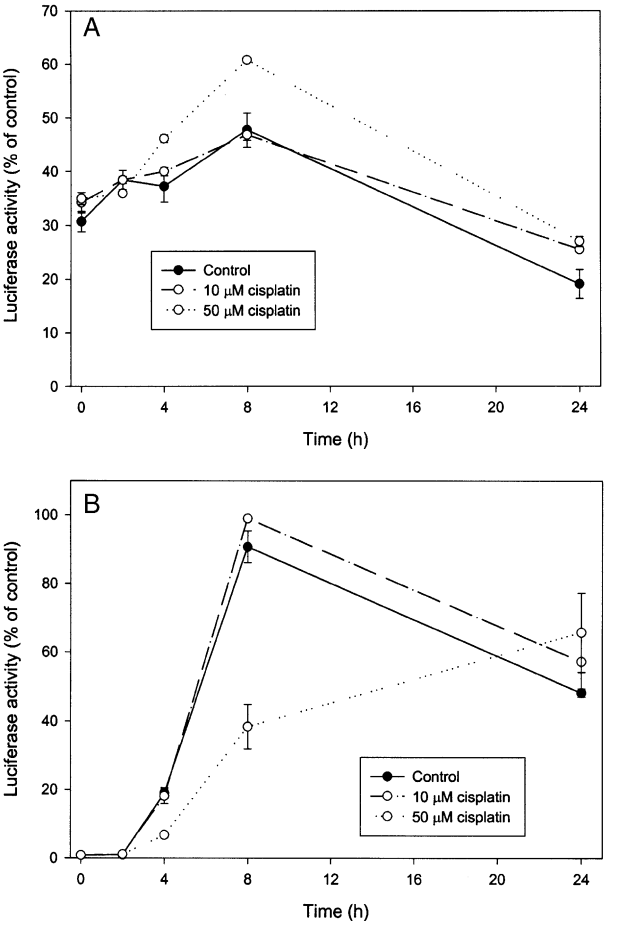

Figure 2. Luciferase time course for the CMV and MMTV promoters. (A) EG109 cells containing and the integrated CMV–luciferase construct were grown for 24 h with or without cisplatin and their luciferase activity assayed. A representative experiment is shown $\pm$ standard error. (B) EG112 cells containing and the integrated MMTV–luciferase construct were grown for 24 h with or without cisplatin and their luciferase activity assayed. Luciferase activity is plotted as the percentage mean of two independent experiments. Standard error is plotted as a percentage of the mean.

the MMTV promoter with dexamethasone. The addition of 10 μM cisplatin did not substantially alter the luciferase activity compared to the control. However the addition of 50 μM cisplatin decreased luciferase activity at both the 4- and 8-h time points. This decrease in luciferase activity was assumed to be due to the inhibition of transcription from the MMTV–luciferase construct by DNA–cisplatin adducts.

From the results of these two time courses and their responses to cisplatin, it was decided to continue this experimental method and assay luciferase activity 6 h after a 2 h exposure to the alkylating agents. For the EG112 cell line, dexamethasone would be added immediately after removal of the alkylating agents.

The effect of alkylating agents on luciferase activity driven by the CMV promoter

None of the alkylating agents tested decreased the CMV luciferase activity below what was observed for the DNA and RNA synthesis (Figure 3). There was a dose–response effect seen for all compounds although it was weak for **5** and absent at high concentrations of cisplatin and **6**.

A trend was observed specific to the major and minor groove binders. The major groove-binding compounds (cisplatin, chlorambucil, **3** and **4**) elevated luciferase activity above the control activity, while all the minor groove-binding compounds (**5**, **6** and **7**) decreased luciferase activity to less than the control.

The effect of alkylating agents on luciferase activity driven by the MMTV promoter

All the alkylators tested had some effect on luciferase activity driven by the MMTV promoter (Figure 4). For chlorambucil and the CBI minor groove binder **7**, luciferase activity increased above the control activity, with a decrease seen only at the highest dose of **7**. For the intercalator-targeted alkylators **3** and **4**, the lower doses increased luciferase activity above the control value while all higher doses decreased luciferase activity. The decreases were similar to those observed for DNA and RNA synthesis inhibition.

Cisplatin and the minor groove targeted mustards **5** and **6** decreased luciferase activity at all the doses tested. For cisplatin the decrease was not substantially different for that observed for DNA and RNA synthesis, while for **6** the decrease was similar to that observed for RNA synthesis. For **5** the luciferase activity was substantial less than both the RNA and DNA synthesis activity at all doses tested.

Discussion

We have explored the effects of seven well-characterized DNA alkylating agents on reporter gene expression from two viral promoters. This was to investigate if DNA alkylating agents could modulate gene expression in an *in vivo* setting. This was a model system and was not designed to investigate a specific cellular or cancer pathway. We used an integrated promoter–reporter gene construct (rather than transient transfection) to mimic the cellular chromosomal expression, replication and repair pathways as much as possible. Furthermore, by studying the expression of the same gene with different promoters we could exclude the effect of drug–DNA alkylation specificity on different open reading frames. The CMV and MMTV promoters were used as they have substantially different mechanisms of regulation and share no significant sequence similarity. The human CMV immediate early promoter/enhancer is from the herpesvirus family with a double-stranded DNA genome. It has been shown to contain transcription factor binding sites for ATF/CRE, NF-κB and AP-1.[20] The promoter can be stress activated by MAP kinases, probably through increased activity of the transcription factors c-Jun and ATF-2.[19] The MMTV promoter is from a B-type retrovirus with a single-stranded RNA genome, and is regulated by steroid hormones through steroid response elements[21] and tissue-specific transcription factors.[22] Basal regulation is controlled by nuclear factor I (NFI) and the octamer-binding factor (OTF-1).

Due to the complex nature of the interactions between DNA binding agents, gene expression and DNA repair, it is challenging to draw strong conclusions from the results of these experiments. However, clear trends were visible in the results and these are suggestive of underlying mechanisms. The luciferase activity from the CMV and MMTV promoters showed considerably different patterns of regulation both with and without drug addition. In no case did the CMV–luciferase activity mimic the MMTV-driven luciferase activity. It is possible that this differential regulation may have been due to differences in the site of genomic integration of the promoter-reporter gene constructs. However, because both CMV and MMTV are considered strong

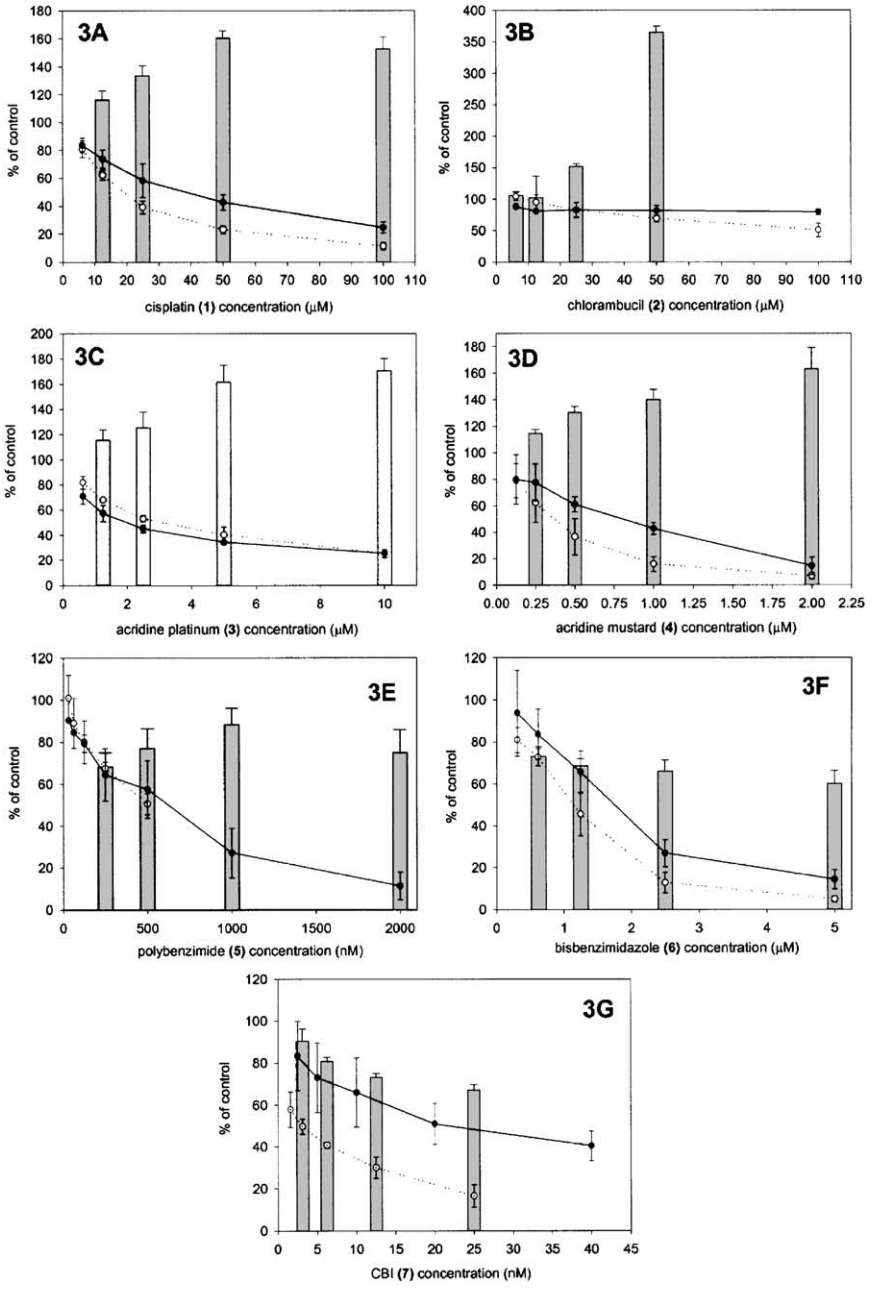

Figure 3. The luciferase activity from EG109 in combination with macromolecular synthesis. DNA synthesis ([^{3}H]thymidine incorporation) is plotted as a dotted line, while RNA synthesis ([^{3}H]uridine incorporation) is plotted as a solid line. Luciferase activity (gray bars) is plotted against alkylator concentration. For DNA and RNA synthesis and luciferase activity the standard error from at least two independent experiments is shown as a percentage of the mean.

promoters in the literature we consider this unlikely. There is also the possibility that the compounds were binding to either the luciferase transcript or protein and this may have modulated the luciferase activity rather than then inhibition of transcription. While the current work does not exclude this possibility, this seems unlikely given the current understanding of the mode of operation of the compounds and their strong binding preference for DNA over RNA or protein.

None of the compounds tested were able to inhibit CMV–luciferase activity beyond 60% of the control. However a trend was apparent within the series of compounds tested. The major groove binders/intercalators all increased CMV–luciferase activity above background, while minor groove binders all

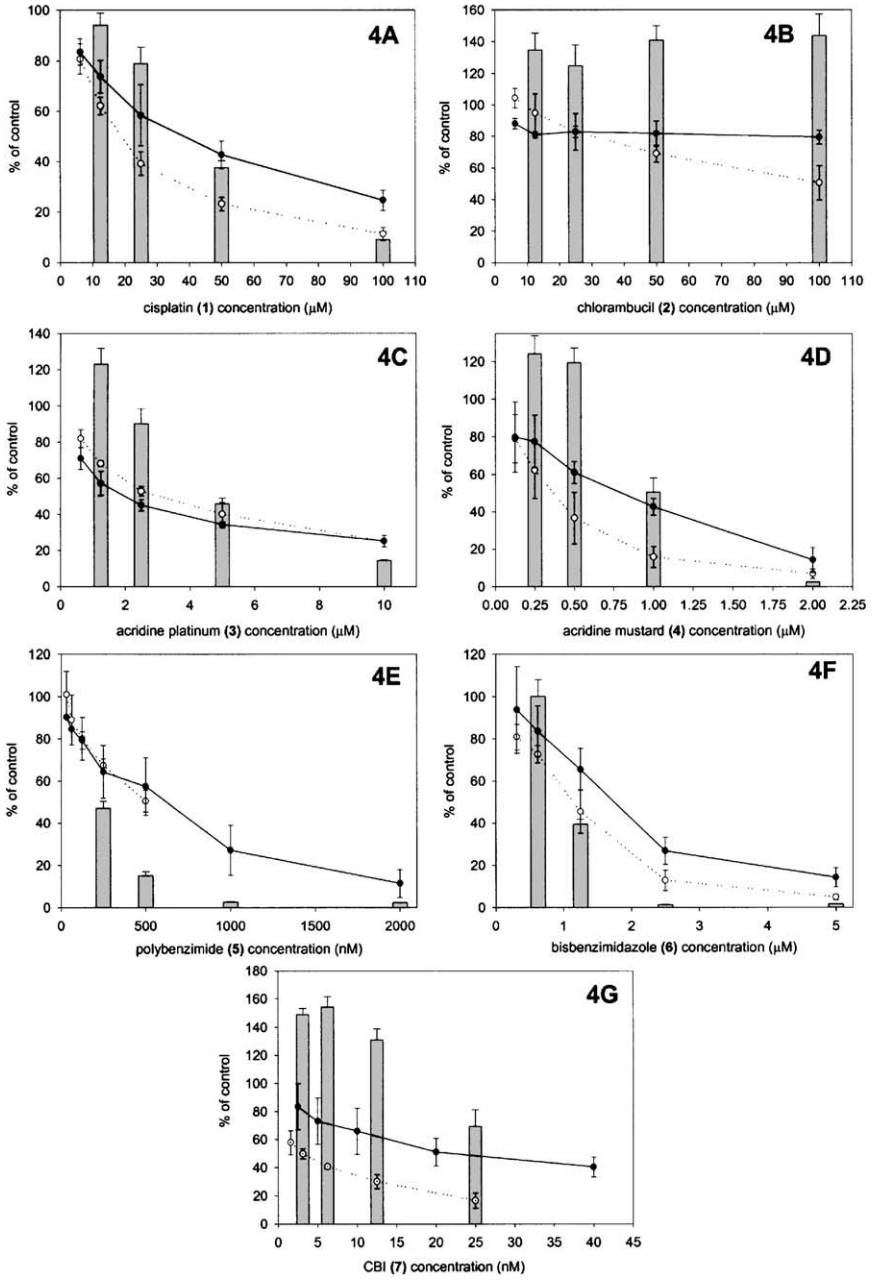

Figure 4. The luciferase activity from EG112 in combination with macromolecular synthesis. DNA synthesis ([^{3}H]thymidine incorporation) is plotted as a dotted line, while RNA synthesis ([^{3}H]uridine incorporation) is plotted as a solid line. Luciferase activity (gray bars) is plotted against alkylator concentration. For DNA and RNA synthesis and luciferase activity the standard error from at least two independent experiments is shown as a percentage of the mean.

decreased CMV–luciferase activity, but not in a dose-dependant fashion. Differential effects between intercalators and minor groove binders have been shown by others.[17,16] The most obvious distinction between the two groups of compounds tested here was that the major groove binders/intercalators all alkylate primarily at guanine bases, while the minor groove binders alkylate only adenine bases. This may suggest that guanine adducts activate the stress responses to a greater extent than adenine adducts, increasing expression from the CMV promoter. Alternatively, the adenine adducts were more difficult to repair than guanine adducts, reducing gene expression.

The differential luciferase activity seen between the two luciferase constructs could be due to a variety of promoter-specific effects. The two promoters recruit different transcription factors resulting in protein–DNA-specific interactions that vary in both strength and sequence specificity. The ability of the DNA alkylating agents to interfere with this interaction may be largely responsible for the variations in luciferase activity. The differences may also reside in the fact that adducts were established on the MMTV promoter before it was induced. As the CMV promoter is highly induced with stress at the time of drug addition (see Results), the DNA adducts were unable to form or were cleared rapidly from the promoter–reporter gene construct. It seems reasonable that a cell's ability to activate and transcribe important genes at a time of stress is essential. Therefore, genes downstream of stress-activated promoters may be preferentially selected for DNA repair. Chemical modified bases that cause a distortion in the double helix are principally repaired by nucleotide excision repair (NER).[23] At has been shown in *Saccharomyces cerevisiae*[24] that environmental stress will activate NER. Also NER has recently been linked to the MAP kinase pathway which is upregulated by cellular stress.[25] Given the conditions that activate the CMV promoter it is conceivable that it is targeted for preferential DNA repair with stimulated NER.

The dual alkylators/minor groove binders (**5** and **6**) inhibited the MMTV–luciferase activity to a greater extent than any of the other alkylators did. The ability of the minor groove binder with spatially separated alkylating units (**5**) to inhibit MMTV–luciferase activity to a greater extent than any of the other DNA alkylating agents is intriguing. It is unknown whether this is due to sequence selectivity of the compound itself to the MMTV promoter or the type of DNA adducts the alkylator forms.

Conclusion

The results from this paper suggest that DNA alkylating agents can substantially modulate the expression of genes within a cell in a differential manner. Gene expression may be both increased and decreased by the addition of DNA alkylators in a manner dependant on both the alkylating agent and the DNA sequence. Furthermore, differences in the promoter sequence and not only the open reading frame can be responsible for the differential regulation.

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