

Effects of DNA Threading *Bis*(9-aminoacridine-4-carboxamides) on Global Gene Expression

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Abstract. The capacity of series of DNA-threading *bis*(9-aminoacridine-4-carboxamides) comprising ethylmorpholino, ethylpiperidine and *N*-methylpiperidin-4-yl sidechains joined by different linkers, to modulate gene expression in human leukaemia cells was investigated. The chosen compounds provided the opportunity for probing the relationships between the structure ligand structure and the drug effects on transcription, information that might lead to a greater understanding of their potential as antitumour agents. As revealed by DNA microarray analysis of 6000 genes, at equitoxic doses, 5×IC₅₀ values for growth inhibition, all of the drugs perturb transcription, resulting in both up- and down-regulation of many hundreds of genes, 24 h after drug exposure. Under these conditions, the capacity to inhibit transcription decreases in the order C3NC3 morpholino > C2pipC2 morpholino > C8 piperidine > C8NMP > C2pipC2 piperidine. Cluster analysis segregated the examined agents into two groups: the first included C2pipC2 morpholino and C3NC3 morpholino and the second C2pipC2 piperidine, C8 piperidine and C8NMP. This classification agreed with the ontological analysis for the markedly up-regulated genes that showed a relatively specific profile for each group. Interestingly, the general up-regulation responses for the first group (C3NC3 morpholino and C2pipC2 morpholino) indicated marked up-regulation amongst the transcription gene set, which suggests that the transcription machinery is the main target for the members of this group. While in the second group (C2pipC2 piperidine, C8 piperidine, C8NMP), the general up-regulation responses for the three agents are dominated by

the protein modification process ontological class, implying at least involvement of topoisomerase poisoning in their mode of action.

Cytotoxic DNA-intercalating agents inhibit cell growth by two well-established mechanisms: poisoning topoisomerases and inhibiting transcription (1). The majority of intercalating cancer drugs in clinical use are of the former type, with examples including adriamycin and mitoxantrone (2, 3). Intercalating inhibitors of transcription are exemplified by the antitumour antibiotics actinomycin, echinomycin and nogalamycin (4, 5). A characteristic shared by these agents is that they bind to DNA reversibly, placing bulky groups in the DNA minor groove covering 4 to 6 base pairs, and form long-lived complexes (6, 7). This ability to form slowly dissociating DNA complexes with half lives of hundreds of seconds, distinguishes the transcription elongation inhibitors from the topoisomerase poisons, which generally dissociate from DNA rapidly in the msecond to second time range (5, 8). Inspired by the above, Wakelin and colleagues have combined the *bis*intercalation motif of echinomycin with the threading mode of nogalamycin, to synthesize a series of *bis*(9-aminoacridine-4-carboxamides) linked by the 9-position by various linkers, in which the carboxamide sidechains comprise ethylmorpholino, ethylpiperidino, and *N*-methylpiperidin-4-yl (NMP) (9). The general design of these agents is predicated upon the notion that simple *bis*intercalating agents can be equipped with threading sidechains that function as "barbed spears", and that with linkers sufficiently long to sandwich two base pairs. The compounds were designed to *bis*intercalate into DNA, sandwiching two base pairs and to thread their sidechains through the helix to form hydrogen-bonding contacts with the O⁶/N⁷ atoms of guanine in the major groove: a hydrogen bonding scheme observed in DNA complexes of the 9-amino-[*N*-(2-dimethylamino)ethyl]acridine-4-carboxamide monomer (10). The threading sidechains were intended to

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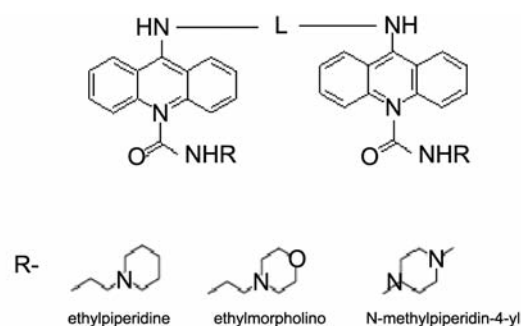
Table I. Cytotoxicities, cell cycle effects and helix unwinding angles of the C3NC3 morpholino, C2pipC2 morpholino, C8 piperidine, C2pipC2 piperidine, and C8NMP.

Compound	Cytotoxicity IC ₅₀ (nM)	Helix unwinding angle	G ₂ /M arrest
C3NC3 Morpholino	47±10 ^a	29±3 ^a	No ^a
C2pipC2 Morpholino	290±150 ^a	27±2 ^a	No ^a
C8 Piperidine	99±24 ^b	20±3 ^b	No ^b
C2pipC2 Piperidine	130±5 ^b	25±1 ^b	No ^b
C8NMP	130±25 ^b	19±3 ^b	yes ^b

a: Data taken from (9); b: data taken from (11).

slow the dissociation kinetics of the DNA-ligand complexes to values approaching those of the natural products, thereby endowing diacridines with the capacity to inhibit transcription by blocking the passage of RNA polymerase II. The rate of dissociation of the *bis*intercalating agent-DNA complexes was slowed by many orders of magnitude compared to the simple diacridines (Wakelin *et al.*, 2003). This was a consequence of the necessity to simultaneously de-intercalate both chromophores, whose withdrawal was hindered by the barbed spear character of the threading sidechains. The threading dimers were cytotoxic to human leukaemia and solid tumour cells, and potency depended upon both linker and sidechain structure (9, 11). Interestingly, some of these agents perturbed the cell cycle, blocking cells in the G₂+M phase, whereas others did not (9, 11) which has been interpreted to suggest that some of the threading dimers may involve topoisomerase activity in their mechanism of action (9, 11).

Microarray determination of global gene expression may provide information about toxicants (14). In this context, studies have addressed radiation (15-17) diverse toxins (18) and a variety of cytotoxic drugs (19, 20). Investigation of the ability of the intercalating threading agents to modulate transcription in the human lymphoblastoid T-cell leukaemia cell line CCRF-CEM, using DNA microarrays, forms a major part of this report. Previous studies of chemotherapeutic agents have almost all involved exposure times of 24 h, either as the only treatment time, or amongst those used. Our studies were designed to further expand this data base. In this work, we have taken the opportunity of sampling ligand-induced changes to transcription profiles with different linker and sidechains. Out of the many *bis*intercalating agents designed, we have chosen five threading agents that compromise three different sidechains and three different linkers were chosen to probe the relationships between ligand structure and drug effects on transcription



Linker	Linker name	Flexibility
(CH ₂) ₈	C8	Flexible
(CH ₂) ₃ NH(CH ₂) ₃	C3NC3	Flexible
(CH ₂) ₂ N(CH ₂ CH ₂) ₂ N(CH ₂) ₂	C2pipC2	Semi-rigid

Figure 1. Structure of the threading diacridines. Compounds are described in the text by reference to their linker and sidechain structure.

Materials and Methods

Cell lines. CCRF-CEM, a human lymphoblastoid T-cell leukaemia cell line was maintained in complete medium consisting of RPMI medium 1624 (Gibco BRL, CA, USA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin/streptomycin, and 10% foetal bovine serum (Trace scientific Ltd, Sydney, Australia) at 37°C, in the presence of 5% CO₂. Cultures were passaged twice weekly at which point they were at 70% of their maximum permissible cell density. The CCRF-CEM cells were seeded at a density of 1×10⁵ cells/ml in T-75 tissue culture flask (Corning, MA, USA) and incubated for 24 h before drug treatment with 5×IC₅₀ concentrations for 24 h. The treatment compounds and their cytotoxicity, cell cycle effects and helix unwinding angles are listed in Table I and their structure is shown in Figure 1. The treatment was performed on three occasions for each drug. Control cells were treated in the same way, except they did not received the drug treatments. Following the treatment periods, the cells were harvested by centrifugation at 1200 rpm at 4°C for 5 min. The culture medium was removed by aspiration, the cells washed with ice-cold PBS, and the pellets recovered again.

RNA isolation and cDNA synthesis and probe labeling. the total RNA was extracted using the acid guanidinium thiocyanate/phenol method (12). The RNA quality was assessed by determining the A260/280 ratio by spectrophotometry and 3 µg RNA was loaded onto a 1% denaturing agarose subject to electrophoresis followed by staining with ethidium bromide. Gel images were taken and the intensity of 28S and 18S ribosomal RNA bands determined by pixel density with the 28S:18S ratio. The RNA samples were stored at -80°C until used for cDNA synthesis. Using RNA from treated and untreated cells, the synthesis of cDNA was initiated by mixing 1.25 µg of oligo dT primers with 20 µg total RNA in a volume of 15.5 µl using diethylpyrocarbonate (DEPC)-treated water and incubated at 70°C for

Table II. Distribution statistics ($\log_2 R/G$ values) for global gene effects of C3NC3 morpholino, C2pipC2 morpholino, C8 piperidine, C2pipC2 piperidine, and C8NMP.

	C3NC3 Morpholino	C2pipC2 Morpholino	C8 Piperidine	C2pipC2 Piperidine	C8NMP
Mean	-0.45	-0.38	-0.20	-0.11	-0.16
Median	-0.22	-0.21	-0.07	-0.03	-0.09
Standard deviation	1.21	1.12	1.11	1.27	1.12
Maximum	-6.10	-7.44	-6.56	-6.78	-6.26
Minimum	5.72	6.25	7.99	8.22	4.75
Skewness	-0.71	-0.72	-0.50	-0.04	-0.52
(a) No. of genes with $\log_2 R/G > 1.5$	131	137	223	294	297
(b) No. of genes with $\log_2 R/G < -1.5$	1025	740	634	521	605
a + b	1156	877	857	815	902
a/b \times 100%	13%	19%	35%	56%	49%

10 min. The incubation mixture was then chilled on ice for an additional 10 min, and mixed with a reaction cocktail consisting of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 500 μ M each of dATP, dCTP and dGTP, 300 μ M dTTP, 200 μ M 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (aminoallyl-dUTP), 10 μ M 1,4-Dithiothreitol (DTT), 150 U of Superscript II, and DEPC-treated water, all in a final volume of 30 μ L. The mixture was incubated at 42°C for 2 h, at which point the reaction was stopped by the addition of 10 μ L of 1 M NaOH followed by 10 μ L of 0.5 M EDTA, which also served to hydrolyse the RNA, with a subsequent incubation for 15 min at 65°C. The reaction mixture was then neutralised by the addition of 25 μ L of 1 M Tris (pH 7.4) which was subsequently removed by passage through a Microcon-30 concentrator (Millipore, MA, USA). The final eluate of approximately 50 μ L was dried in a vacuum centrifuge. The amino-allyl-substituted cDNA was resuspended in 9 μ L of 0.1 M sodium bicarbonate buffer, pH 9.5, and allowed to sit at room temperature for 15 min to ensure complete dissolution. The samples were then mixed with Cy5 (red fluorescence) or Cy3 (green fluorescence) dye and incubated for 1 h at room temperature in the dark, the coupling reaction being quenched by the addition of 4.5 μ L of 4 M hydroxylamine followed by incubation for a further 15 min at room temperature. Unincorporated dye was removed from the cDNA preparations by passage through a QIAquick PCR purification column according to the manufacturer's instructions (Qiagen, Hilden, Germany). The final samples were eluted from the column with 50 μ L of sterile water.

Microarray hybridization, washing and fluorescence imaging. The cDNA microarray consisted of 5705 sequence-verified known human genes and 420 control genes, some human, some yeast, spotted on to Telechem® slides (Genetix, New Milton, UK) as duplicate features (total of 12250 spots). The chips were purchased from the Ramaciotti Centre for Genome Function at the University of New South Wales. A list of the 5705 genes is to be found at the GeneSpring Workgroup held at the Garvan Institute for Medical Research, Sydney, Australia (<http://gspring.garvan.unsw.edu.au>). Following hybridization, the microarray was washed in a pre-warmed (50°C) 1 \times saline sodium citrate (SSC) solution containing 0.03% sodium dodecyl sulfate (SDS) for 5 min followed by successive 5 min washes in 0.2 \times SSC and 0.05 \times SSC at room temperature. The microarrays were scanned on an Axon II Scanner

with a multi-channel image generated which was subsequently analysed with Genepix software (Axon-Molecular Devices, Sunnyvale, CA, USA).

Data analysis. Each full experiment, starting from drug treatment and culminating in hybridization of fluorescently-labelled cDNA to the microarray, was performed on three separate occasions, thereby generating three fully independent microarray data sets for all the compounds studied. For every feature on each array, the fluorescence intensity of the Cy5-(Red, R) and Cy3-(Green, G) labelled cDNA was determined from the scanned array images, after equalization of fluorescence emission intensity for each dye, and subtraction of background fluorescence. Gene expression values for each feature were expressed as the base 2 logarithm of the ratio of Cy5 (R) intensity to Cy3 (G) intensity ($\log_2 R/G$). Using the Bioconductor software, the $\log_2 R/G$ was normalized across each microarray using a "print-tip lowess" computation. This procedure produces a list of the relative intensities, and hence relative expression level, for each of the experimental genes detected in both channels. For each of the three normalized microarray data sets for each drug treatment, in which every gene had a duplicated spot per array, the median $\log_2 R/G$ for all 6 expression measurements were determined for each gene. The distributions of the $\log_2 R/G$ values were plotted as histograms and examined using standard descriptive statistics (mean, median, skewness, and kurtosis), so allowing comparison of the global effects on gene expression of the drugs studied. The missing values were estimated by the K-nearest neighbor (KNN) approach. When investigating whether drugs had similar or different effects on transcription, all the genes whose absolute variation in level of expression is by a factor of 3-fold, as revealed by those genes whose $\log_2 R/G$ is $\geq \pm 1.5$ were compared. All the genes on the array were subjected to unsupervised hierarchical clustering, the extent of the similarity and differences in expression profiles between drug treatments being based on the correlation coefficient used within the software Cluster and displayed as heat maps using TreeView (13). The gene ontology database was interrogated *via* the web-based application DAVID (<http://david.abcc.ncifcrf.gov>) which provides for ontology mapping, annotation, and visualization of results. A level 5 search in the "biological process" category was carried out to provide the highest degree of specificity for the target function.

Results

The impact of each drug on the global gene expression, as shown by the distributions of the \log_2 R/G values using standard descriptive statistics is presented in Table II. The \log_2 R/G values for each drug were summarized in histogram format relating the extent of perturbation in the gene to the number of genes so affected (Supplementary Figure 1). The histograms show that the expression levels could remain unchanged, be elevated or be diminished. All five histograms share negative means, medians, and skewness values, which indicate that the number of genes in the down-regulated region was greater than the number in the up-regulated region. The two morpholino dimers exhibited the most negative mean, medium, and skewness values and exhibited the lowest number of up-regulated genes. This superiority in the inhibitory effect was clearly seen in the higher number of genes that were down-regulated 3-fold or more. Comparing the ratios between the up- and down-regulated genes for the five agents, which were 13, 19, 35, 56, and 49% for C3NC3 morpholino, C2pipC2 morpholino, C2pipC2 piperidine, C8 piperidine and C8NMP, respectively, reinforces the same finding that the morpholino compounds had a greater inhibitory effect on global gene expression than the other compounds.

Commonality between the five agents. The extent of commonality between the particular genes exhibiting the most marked change in expression is shown in Table III based on \log_2 R/G ≥ 1.5 . Pair-wise comparisons between the down-regulated genes, showed that C2pipC2 morpholino and C3NC3 morpholino shared the highest percentage of commonality with 417 genes, accounting for 25% of the sum of the genes down-regulated 3-fold or more by these two agents. The second highest percentage shared was C8 piperidine and C8NMP, accounting for 23% of the sum of their down-regulated genes. The number of shared genes, 324, surpassed those showed by C8 piperidine and C2pipC2 piperidine by 83, showing the importance of the linker in determining the impact on global gene expression. For the up-regulated genes, the highest commonality of 86 shared genes was exhibited by the two piperidine dimers, which accounted for 17% of the sum of the genes up-regulated 3-fold or more by these two agents, followed by 75 common genes shared by the C8-linked dimers. The two morpholino compounds shared a relatively low percentage between them and exhibited different profiles, in which C2pipC2 morpholino shared higher percentages of common genes with the other three agents.

Extent of similarities and differences between the threading dimers. Unsupervised hierarchical cluster analysis of the 5704 genes, the total number of genes on the array, confirmed the relationships between the agents studied. Figure 2 shows the

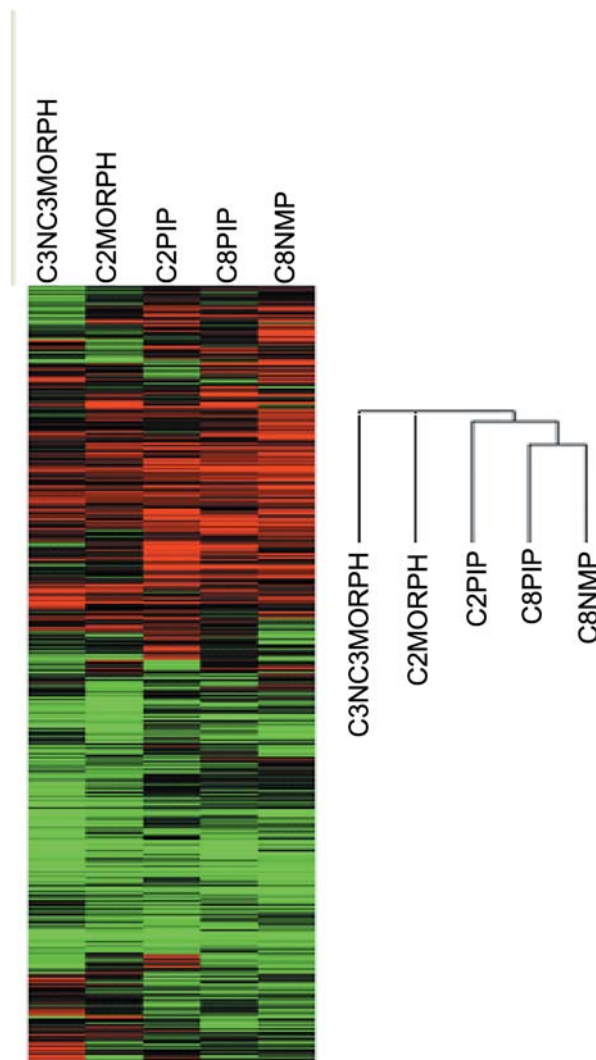


Figure 2. Cluster analysis for C3NC3 morpholino, C2pipC2 morpholino, C8 piperidine, C2pipC2 piperidine, and C8NMP for all genes on the array (5704).

behaviour of the 5704 genes as is printed in the array. This analysis has the advantage of demonstrating all possible relationships between the examined agents at once. Although the heat map implied a great degree of similarity amongst the transcriptional responses for all five agents examined, it graphically illustrated that the five compounds segregated into two groups, one containing C2pipC2 piperidine, C8 piperidine and C8NMP, and the other including the two morpholino dimers, as clearly shown in the supporting dendrogram (see Figure 2). Interestingly, within the three component group, the heat map showed that the similarity between C8 piperidine and C8NMP was more than that between the two piperidine compounds. This similarity between the two compounds was

Table III. Pair-wise commonality for those genes elevated or repressed by expression level changes ≥ 3 -fold. Percentage: shared genes expressed as a fraction of the sum of the genes that were up- or down-regulated 3-fold or more by the two agents included in the combination.

Combination	No. of common down-regulated genes	No. of common up-regulated genes
C3NC3 Morpholino + C2pipC2 morpholino	417 (25%)	18 (10.7%)
C3NC3 Morpholino + C8 piperidine	375 (22.6%)	11(3.1%)
C3NC3 Morpholino + C2pipC2 piperidine	269 (17.0%)	11(3.1%)
C3NC3 Morpholino + C8NMP	336 (20.0%)	14 (3.3%)
C2pipC2 Morpholino + C8 piperidine	258 (18.6%)	38 (10.5%)
C2pipC2 Morpholino + C2 pip C2 piperidine	217(17.2%)	37 (8.6%)
C2pipC2 Morpholino + C8NMP	273 (20.3%)	33 (7.6%)
C8 Piperidine + C2pipC2 piperidine	241(20.9%)	86 (17.0%)
C8 Piperidine + C8NMP	324(23.0%)	75 (14.4%)
C2pipC2 Piperidine + C8NMP	220(19.0%)	69 (11.7%)

Table IV. Gene ontology groups for up-regulated genes (≥ 3 -fold) for C3NC3 morpholino, C2pipC2 morpholino, C8 piperidine, C2pipC2 piperidine, and C8NMP. Minimum entry, 10 genes. A is the number of genes observed in each ontology group for each agent. B is the percentage fraction of the total number of genes perturbed ≥ 3 -fold by each agent.

Ontological class	C8NMP		C2pipC2 Piperidine		C8 Piperidine		C2pipC2 Morpholino		C3NC3 Morpholino	
	A	B	A	B	A	B	A	B	A	B
Protein modification process	36	0.14	38	0.14	34	0.16	NA	NA	NA	NA
Regulation of transcription, DNA-dependent	NA	NA	NA	NA	NA	NA	27	0.21	21	0.19
RNA biosynthetic process	NA	NA	NA	NA	NA	NA	27	0.21	21	0.19
Apoptosis	26	0.10	NA	NA	NA	NA	12	0.09	NA	NA
Protein kinase cascade	18	0.07	16	0.06	13	0.06	NA	NA	NA	NA
Intracellular transport	NA	NA	19	0.07	NA	NA	11	0.09	NA	NA
Protein transport	NA	NA	18	0.07	NA	NA	10	0.08	NA	NA
Regulation of progression through cell cycle	17	0.06	NA	NA	14	0.06	9	0.07	NA	NA
Regulation of transcription from RNA polymerase II promoter	16	0.06	NA	NA	11	0.05	10	0.08	NA	NA
Establishment and/or maintenance of chromatin architecture	10	0.04	NA	NA	NA	NA	NA	NA	NA	NA
Negative regulation of cell proliferation	8	0.03	9	0.03	11	0.05	NA	NA	NA	NA

confirmed in the dendrogram, in which these two agents were placed together in a subgroup leaving the C2pipC2 piperidine alone in the other subgroup.

Ontological analysis. The ontological analysis for the genes that were up-regulated more than 3-fold identified twelve biological classes containing ten or more entries in at least one treatment (Table IV). Although there was a notable degree of unique behavior for each agent, this analysis, once again, segregated the agents into two groups, each with a distinguished ontological profile. The first profile can be seen in the C8 piperidine, C2pipC2 piperidine and C8NMP cases, and is characterized by having the protein modification process as the most represented class. The genes within this ontological class accounted for 14, 14 and 16% of the genes up-regulated by C8NMP, C2pipC2 piperidine and C8 piperidine, respectively. These three agents exhibited other clear commonality in having similar representation for

protein kinase cascade and negative regulation of the cell proliferation ontological classes. Importantly, these three agents did not have any representation in the regulation of transcription ontological class, which was the most represented ontological class for the C3NC3 morpholino and C2pipC2 morpholino cases. The second ontological profile was exemplified by C3NC3- and C2pipC2-morpholino. The most obvious commonality between these two agents was in having the regulation of transcription and the RNA biosynthetic process ontological classes as the most populated groups, with 19% of C3NC3 morpholino and 19% of C2pipC2 morpholino up-regulated genes.

Discussion

The experiments described here are the first to probe the transcriptional properties of the *bisintercalating* threading dimers. Both up- and down-regulation of mRNA was shown

by all the agents studied (supplementary Figure 1 and Table II). Out of the 5704 genes detected, 877 with C2 morpholino, 1156 with C3NC3 morpholino, 857 with C8 piperidine, 815 with C2pipC2 piperidine and 902 with C8NMP, showed changes in expression ≥ 3 -fold, indicating that these threading *bis*intercalating agents produced substantial modifications to transcription. All five profiles had negatively skewed values and a substantially higher number of down-regulated genes, reflecting pronounced inhibitory effects. Having regard to both the number of genes down-regulated (Tables II) and the extent of inhibition (Figure 2), at equitoxic doses and at equivalent exposures, the capacity to inhibit transcription decreases in the order C3NC3 morpholino > C2pipC2 morpholino > C8 piperidine > C8NMP > C2pipC2 piperidine. Regarding the up-regulation responses, an almost opposite order was seen, however, here the two morpholino dimers up-regulated a notably lower number of genes than C2pipC2 piperidine, C8NMP and to lesser extent C8 piperidine. Taking the up- and down- responses together, the examined five agents fall into two major groups, the two morpholino dimers in the first and the other three in the second. Direct comparisons between the most affected genes indicated a relatively high degree of concordance within the two groups. Which was also illustrated by the hierarchical clustering and the associated dendrogram. This division clearly signaled that the sidechain had a major role in determining the transcriptional effect, although the linker had some influence on transcription responses within the distinguished groups.

The closer behavior of the two morpholino dimers and their higher inhibitory impact on gene expression might be explained by the crystallographic studies of monomeric 9-aminoacridine-4-carboxamides with dimethylaminoethyl and ethylmorpholino sidechains, which established that the compounds bind to DNA with their carboxamide sidechains lying in the major groove, forming hydrogen bonds with the O⁶/N⁷ atoms of guanine. In these structures, the ethylmorpholino sidechain, being more bulky, covers two base pairs, in contrast to the *N,N*-dimethylaminoethyl sidechain, which extends just to the bonding guanine (10, 21). This more bulky coverage with the morpholino monomers would result in more kinetic stability for the ethylmorpholino monomer and DNA complex. Applying this at the dimer would explain the higher level of transcriptional inhibition seen in this study. The observed differences in responses amongst the two morpholino dimers might be due to the flexibility of the linker exemplified in the C3NC3, which has a strong influence in slowing dissociation rates, and presumably in enhancing affinity (9, 22). Of course, the subtle differences in transcriptional response between the semi-rigid- and flexible-linked dimers, at the specific gene level might at least have part of their origins in different specificities amongst the target binding sites, since these

bulky linkers undoubtedly have the capacity to distinguish between sandwiched AT and GC dinucleotide pairs in the *bis*intercalated complex.

Replacing the morpholino group, pK_a 8, with the more basic piperidine moiety, pK_a 11, which strengthens the hydrogen bond energy (11), seemed not to have the desired impact on global gene expression of strengthening the affinity of the piperidine compounds toward the DNA. This finding agreed with our previous publication (11), in which we concluded that although the ethylpiperidino series could form stable complexes with circular DNA on agarose gels, the helix unwinding angle measurements values were low for typical *bis*intercalating diacridines. On the other hand, in that study the DNA affinity of the *N*-methylpiperidin-4-yl series was insufficient to make the favorable interactions, intended by the compound design, with the guanine O⁶/N⁷ atoms (11). This would explain the relatively lower inhibitory effect with C8NMP in the presented study. Thus, the barbed spear character of the ethylpiperidino and *N*-methylpiperidin-4-yl seems not to be optimum, which could also account for C8 piperidine and C8NMP appearing in one subgroup and C2pipC2 piperidine in another (see dendrogram in Figure 2). In other words, if the sidechains had the intended barbed spears character, the effect of the sidechain would dominate and determine the impact on gene expression, as seen in the morpholino sidechain situation. However, if the barbed spears function is not fulfilled, the sidechain would not be the main factor, leaving the linker, which is considered to be one of the main players in the intercalation process (22), to be the main determinant.

For more insightful views into the biology of these transcriptional changes induced by the five *bis*intercalating agents, the genes up-regulated 3-fold or more were partitioned into different ontological classes. Significantly, the ontological responses followed the above classification, yielding two biological response profiles. The first, as before shared by C2pipC2 piperidine, C8 piperidine and C8NMP and characterized by the domination of the protein modification process. Assuming the cells up-regulated some functions to offset drug inhibition, a kind of biological Le Chatelier's Principle, and remembering that the *bis*intercalating agents exert their mechanism of action either by inhibiting transcription or by poisoning the topoisomerase enzyme, this implied the involvement of topoisomerase poisoning in the mode of action of C2pipC2 piperidine, C8 piperidine and C8NMP. Although this is a new finding for the piperidine dimers, it was not surprising for the C8NMP that shares the ability to block cells in the G2+M phase with the established topoisomerase poisons as shown in our previous publication (11). By an analogous argument, the domination of the regulation of transcription ontological genes in the second biological response profile implied that C3NC3 morpholino and C2pipC2 morpholino engage apoptosis through the inhibition of transcription.

In summary, the transcriptional responses are strongly dependent on the sidechain structure and, to a lesser extent, on the linker structure, and the agents could be divided into two groups, with C3NC3 morpholino and C2pipC2 morpholino exhibiting more inhibitory impact on gene expression, while the C8 piperidine, C2pipC2 piperidine, and C8NMP exhibit a lower inhibitory effect and a relatively higher up-regulation effect. The main target of the first group (C3NC3 morpholino and C2pipC2 morpholino) is the transcription machinery, while the ontological profiles of the second group (C8 piperidine, C2pipC2 piperidine, and C8NMP) imply at least the involvement of topoisomerase poisoning in its mode of action.

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