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INDUCTION OF THE c-*myc* BUT NOT THE cH-*ras* PROMOTER BY PLATINUM COMPOUNDS

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Abstract—The recombinant plasmids p324, p330 and p323 carrying the aminoglycoside phosphotransferase (*aph*) gene and 5′ flanking c-*myc* sequences linked to the reporter gene chloramphenicol acetyl-transferase (*cat*) were introduced into the mouse erythroleukaemic cell line F412B2TK$^-$ and stable transfectants resistant to geneticin G418 were obtained. The effects of *cis*-platin and two novel platinum compounds, D19466 (lobaplatin) and D17872, on c-*myc* promoter regions were studied using a large range of drug concentrations and correlated with cytotoxicity data. It was found that *cis*-platin and D19466 show a similar pattern of cytotoxicity and activation of c-*myc* promoter-driven *cat* gene expression with maximum effect (increased expression of 7.4- and 8.1-fold, respectively) at a concentration of 5×10^{-5} M, while the less cytotoxic D17872 only slightly activates *cat* expression at the same concentration. However, when the F412B2TK$^-$ cell line was transfected with a plasmid carrying 5′ flanking sequences of the c-H*ras*1 gene with promoter/enhancer function, linked to the *cat* reporter, no similar inductive effect was observed with any of the platinum drugs used. These data suggest that platinum compounds and possibly other DNA-damaging agents may specifically influence the expression of certain genes.

Key words: platinum compounds; c-*myc* oncogene

Platinum co-ordination complexes have been used for many years in cancer chemotherapy [1]. *cis*-Platin**, is widely used in the chemotherapy of several types of human tumours including squamous carcinoma of head and neck, testicular, ovarian, bladder and lung cancer [2, 3]. Extensive evidence implies that DNA is the critical target for platinum cytotoxic effect [4]. Its mode of action has been mainly correlated with the formation of intra- and inter-strand DNA crosslinks and inhibition of DNA synthesis [4]. The aquation hydrolysis rate of platinum complexes is an important factor for their interaction with DNA [5] and therefore the role of the carrier ligand in these drugs may be critical for their cytotoxic effect and pharmacokinetics [6, 7]. New platinum analogues continue to be synthesized with a view to developing non cross resistant, less toxic and more potent drugs. D19466 (lobaplatin) [8] and D17872 [9] (Fig. 1a [10, 11]) are two promising platinum compounds, the former being assessed in phase II clinical trial.

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** Abbreviations: *cis*-platin, *cis*-diammine-dichloro-platinum (II); D19466, *cis*-(*trans*-1,2-cyclobutane-bis-(methylamine) - *N*,*N*′ - (2*S*) - 2 - hydroxy - propanoato - plantin);D17872, *cis*-dichloro-3-(4-chlorophenyl)-1,2-diamino-propan-plantin; CHO, Chinese hamster ovary; MEL, mouse erythroleukaemic; FCS, foetal calf serum; *aph*, aminoglycoside phosphotransferase; MTT, 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density.

The c-*myc* proto-oncogene encodes for two nuclear phosphoproteins p62 and p64 involved in the control of cellular proliferation, differentiation and apoptotic cell death [12, 13]. Altered c-*myc* expression due to proviral insertion, chromosomal translocation and gene amplification has been implicated in the genesis of several types of malignancies in chickens, rodents, rats and humans [14, 15]. The regulation of c-*myc* gene expression is complex and is controlled not only at the level of transcription, by both positive and negative *cis*-acting regulatory elements, but also at a post-transcriptional level [12].

The superfamily of *ras* genes consists of many related genes which encode proteins which share the characteristic of signal transduction. Among them, the H-*ras*, K-*ras* and N-*ras* are the most well-characterized and studied [16]. Genetic defects characterizing the *ras* genes include amplification and loss of a normal *ras* allele, but the commonest alterations involve point mutations in codons 12, 13 and 61 of the three *ras* genes. Overexpression without any obvious genetic alteration has been reported in many naturally occurring and experimentally induced tumours [15, 16].

Scientific interest has been recently focused on the possible role of platinum and other antitumour agents on oncogene expression. UV radiation and alkylating compounds have been shown to induce c-*myc* expression [17]. Jones and colleagues [18] suggested that the preferential gene-specific repair

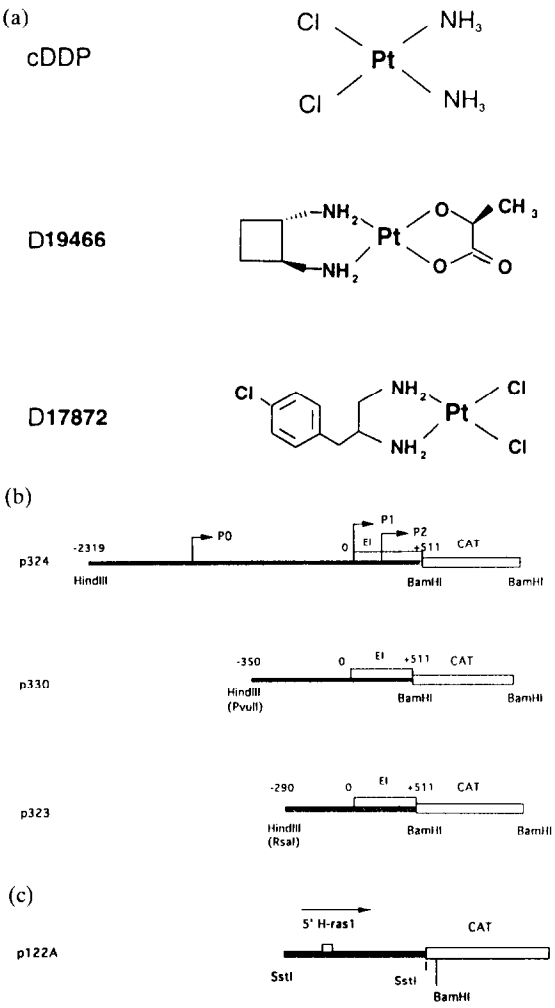

Fig. 1. (a) Molecular structures of *cis*-platin (cDDP), D19466 (lobaplatin) and D17872. (b) A 2830 bp HindIII/BamHI fragment from human c-*myc* containing the exon I (EI) on the c-*myc* oncogene and 5′ flanking sequences was linked to the coding sequence of the *cat* gene. 5′ Terminal deletions of the *myc* promoter were constructed by PvuII or RsaI cleavage followed by HindIII linker addition and similarly linked to the *cat* gene. The plasmid p330 contains a FOS/JUN (AP-1) binding site which has been eliminated in the plasmid p323. The hybrid *myc/cat* constructs were cloned into the multi-cloning site of the vector p304 containing the *aph* gene [10]. (c) The plasmid p122A carries the 800 bp SstI fragment from the normal c-H*ras*1 gene, containing the untranslated exon (open box), linked to the reporter *cat* gene [11]. Although no obvious TATA box exists, there are several RNA polymerase SP1 binding sites and four transcriptional start sites have been identified. This c-H*ras*1 fragment has been shown to contain promoter/enhancer activities [11].

of the *cis*-platin-DNA adducts observed in CHO cells may be a component of platinum drug resistance. One of these genes, the c-*myc* proto-oncogene, has further been shown to contribute to platinum resistance in mouse erythroleukaemic cells [19].

Despite the significance of the c-*myc* gene as a determinant of the cellular response to *cis*-platin and other antitumour agents little is known about the interaction and modulation of the c-*myc* expression by these drugs. In this study we present evidence that *cis*-platin as well as two novel platinum analogues (D19466 and D17872) activate the c-*myc* but not the c-H*ras*1 promoter in a concentration and time-dependent manner.

MATERIALS AND METHODS

Materials. *cis*-Platin (Sigma, Poole, U.K.) and D19466 (Asta, Frankfurt, Germany) were dissolved in 0.9% NaCl and stored at −20°. D17872 (Asta, Frankfurt, Germany) was dissolved in ethanol and used immediately for cell treatment.

The molecular structures of these three platinum compounds are given in Fig. 1a.

Recombinant plasmids and cell lines. The MEL cell line F412B2TK$^-$ [20] used in this study was continuously maintained in Ham's F12 medium (Gibco, Uxbridge, U.K.) with 50 μg/mL streptomycin and 300 μg/mL penicillin, supplemented with 10% FCS (Flow) and 2 mM glutamine, at 37° in a 5% CO_2 atmosphere.

The plasmids p324, p330 and p323 carrying the *aph* gene and 5′ c-*myc* flanking sequences linked to the reporter *cat* gene (Fig. 1b) and the plasmid p122A (Fig. 1c) carrying 5′ flanking sequences from the normal c-H*ras*1 linked to the *cat* gene, were introduced into the F412B2TK$^-$ cell line using the calcium phosphate technique [21, 22] and stable transfectants (F324-1, F330-3, F323-1 and F122A-2, respectively) resistant to geneticin G418 (Sigma, Poole, U.K.) were obtained.

All cell lines were routinely tested for mycoplasma using the mycoplasma detection kit of Boehringer Mannheim (Sussex, U.K.).

Cell treatment and cat *assays.* Exponentially growing cells were treated with various concentrations of platinum analogues (*cis*-platin, D17872 or D19466). After the appropriate time exposure, cells were harvested and assayed for *cat*-activity as previously described [23]. In brief, 100 μg of protein extracts isolated from treated or untreated cells after three cycles of freeze (−70°) and thaw (37°) were mixed with 10 μL of 0.4 M acetylCoA (Sigma, Poole, U.K.) in 1 M Tris–HCl (pH 7.8) and 2 μL of ^{14}C-chloramphenicol (57 mCi/mmol) (Amersham, U.K.) were added. The mixture was incubated at 37° for 90 min and 0.6 mL of ethyl acetate (BDH, Lutterworth, U.K.) was then used to extract the chloramphenicol. The organic layer was dried and taken up in 30 μL of ethyl acetate, spotted on silica gel thin-layer plates (Sigma, Poole, U.K.) and run with chloroform/methanol (95:5, v/v). After autoradiography, spots were cut out and counted in a Packard scintillation counter.

cat-Activity was estimated as pmol of chloramphenicol acetylated per mg of protein per hour of incubation.

Assay for cytotoxicity. Platinum-induced cytotoxicity was estimated using the colorimetric assay of Mosmann [24]. In brief, F324-1 cells were plated on a 96 well plate (Nunc) at a concentration of 1.5×10^4 cells/mL and treated with various

concentrations (1.0×10^{-7}–1.0×10^{-4} M) of platinum analogues for 24 hr. Twenty microlitres of 5 mg/mL MTT (Sigma, Poole, U.K.) in PBS were then added in each well, incubated for 5 hr at 37° and the formazan crystals formed were dissolved in DMSO. The OD was recorded at 550 nm using a Becton Dickinson Multiscan. The IC_{50} values were the drug concentrations inducing 50% reduction in the OD.

Clonogenic agar capillary microassay. The clonogenic microassay for F324-1 was carried out in agar-containing glass-capillaries as described before [25, 26]. Briefly, the cells were cultured at a concentration of 25,000 cells/mL in glass capillaries containing Ham's F12 medium supplemented with 20% FCS and 0.18% Agar (Sigma) in a final volume of 30 μL. Each of the three platinum compounds was added at graded concentrations (1×10^{-7}–5×10^{-5} M). Triplicate capillaries of each of the cultures described above were incubated for 5 days and colonies (aggregates of 20 or more cells) were then counted using a dissecting microscope.

Statistical analysis. Each experiment was performed at least in triplicate. Standard deviation for each mean value in these experiments was then estimated and is presented in histograms as vertical bars. *cat*-Activity values were analysed with Student's *t*-test.

RESULTS

The effect of *cis*-platin and two novel platinum compounds D19466 (lobaplatin) and D17872 (Fig. 1a) on c-*myc* promoter driven *cat*-activity in F324-1, F330-3 and F323-1 MEL cells harbouring the recombinant plasmids p324, p330 and p323, respectively (Fig. 1b), was examined. These lines were chosen on the basis of approximately equal plasmid copy number integrated in the genome of the transfectant cells, as determined by Southern blot analysis (data not shown). As shown in the autoradiograph of Fig. 2a and the histograms of Figs 2b and 3a, the amount of *cat* produced in the cell line F324-1 after 24 hr drug exposure varies with the amount of D19466 and *cis*-platin administered to the cells while it remains practically constant over a wide range (1×10^{-6}–1×10^{-4} M) of D17872 concentrations. At lower drug concentrations (1×10^{-7}–5×10^{-7} M) none of these compounds had any effect on *cat*-activity (data not shown). F324-1 cells respond to D19466 at the concentration of 1×10^{-6} M, while neither D17872 nor *cis*-platin had any effect at the same concentration (Fig. 2 and data not shown). At the optimum concentration of 5×10^{-5} M, the increase in *cat*-activity caused by D19466 is time-dependent with maximum effect (8.1-fold increase, $P < 0.001$) at 24 hr drug treatment (Fig. 3b). At higher doses (1×10^{-4} M D19466) or at prolonged exposure (36 and 48 hr) *cat*-activity was found to decrease (data not shown). Similar results were obtained for *cis*-platin which induces a 7.7-fold increase ($P < 0.01$) in *cat*-activity at a concentration of 5×10^{-5} M at the optimal time period of 24 hr, while D17872 only slightly activates *myc*-driven *cat*-activity (1.6-fold increase, $P < 0.01$) under the same conditions (Fig. 3a). F330-3 and F323-1 cell lines also responded to D19466 at the optimum

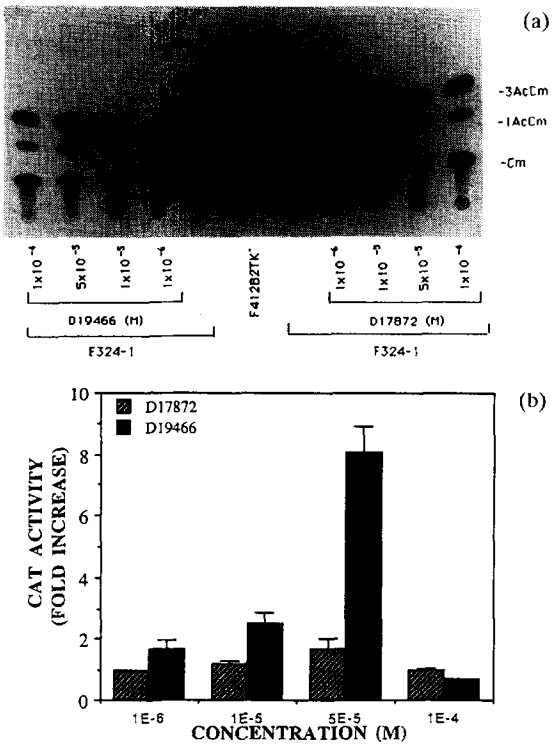

Fig. 2. Induction of c-*myc* promoter-driven *cat*-activity by D19466 and D17872. (a) Chromatogram of representative *cat*-assay with protein extracts from parental F412B2TK⁻ and transfectant F324-1 cells with and without treatment by D19466 (lanes 1–4) and D17872 (lanes 6–10). Cells were exposed for 24 hr to various drug concentrations, harvested and analysed for *cat*-activity as described in the Materials and Methods section. (b) Histogram of recorded *cat*-activities. An average from three independent experiments is given. Standard deviation of six values is given as vertical bars.

concentration of 5×10^{-5} M showing a 4.5 ($P < 0.001$) and 3.1-fold ($P < 0.001$) increase in *cat*-gene expression, respectively (Fig. 3a). In these cell lines *cis*-platin increased *cat* activity by 3.9 ($P < 0.001$) and 2.5-fold ($P < 0.001$) respectively, while D17872 had no significant effect at the same concentration. Induction of c-*myc* promoter-driven *cat* activity was also observed in other F412B2TK⁻ clones tested as well as in K562 human erythroleukemic cells and Rat-1 fibroblasts transfected with the same c-*myc* promoter/*cat* constructs (data not shown).

On the contrary, we observed no effect on c-H*ras* promoter-driven *cat* activity when the cell line F122A-2 was treated with the same range of D19466 or *cis*-platin concentrations for various time intervals (data not shown).

The cytotoxic effect of D19466, D17872 and *cis*-platin was measured by the MTT cell proliferation assay for various time intervals (0, 24, 48, 72, 96, 120 hr) and drug concentrations (1×10^{-7}–1×10^{-4} M). As depicted in Fig. 4a, D17872 inhibits cell proliferation after 24 hr of drug exposure but is less effective than both D19466 and *cis*-platin. Similar results were obtained when we performed a

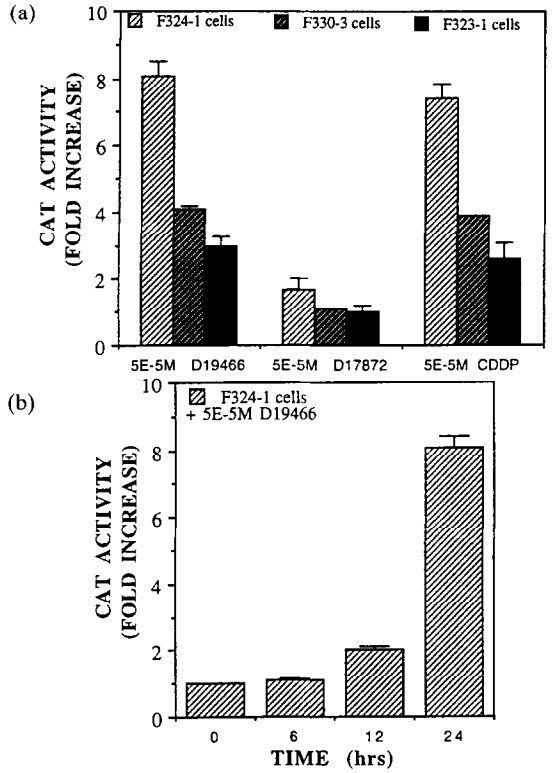

Fig. 3. (a) Induction of different deletions of c-*myc* promoter by D19466, D17872 and *cis*-platin. *cat*-Activity values were compared using the Student's *t*-test: a/b: $P < 0.001$, a/c: $P < 0.001$, b/c: $P < 0.01$, d/e: $P < 0.01$, d/f: $P < 0.01$, e/f: $P > 0.05$ NS. (b) Induction of *cat*-activity in the F324-1 cell line treated with 5×10^{-5} M D19466 at various times post-treatment. Standard deviation is given as vertical bars.

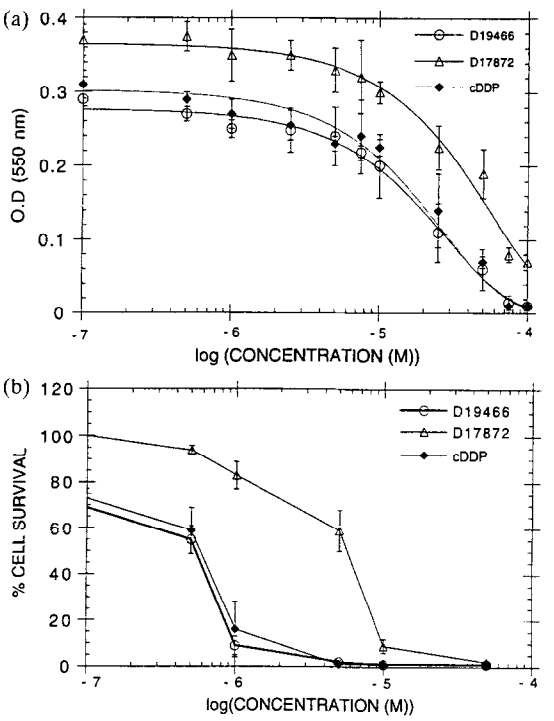

Fig. 4. (a) Cell viability (OD_{550}) in response to D19466, D17872 and *cis*-platin after 24 hr of continuous F324-1 cell exposure to graded drug concentrations. Standard deviation of nine values is presented as vertical bars. The OD_{550} for untreated cells was 0.38 (±0.02). (b) Inhibition of colony growth on day 5 of F324-1 cell exposure to graded concentrations of D19466, D17872 and *cis*-platin. Standard deviation of six values is given.

clonogenic agar capillary microassay. In control cultures, F324-1 cells formed 65 (±6) colonies on day 5. The addition of D19466 decreased the number of colonies in a dose dependent manner, with an IC_{50} of 4.8×10^{-7} M. *cis*-Platin showed a rather similar IC_{50} value (5.2×10^{-7} M) while D17872 was less effective, causing 50% inhibition of colony growth at a concentration of 5.2×10^{-6} M (Fig. 4b).

DISCUSSION

Platinum compounds are widely used as anticancer drugs. They exert their action mainly as a consequence of interaction with DNA and inhibition of DNA synthesis [4, 5]. Nevertheless, the binding of platinum drugs to DNA is not by itself sufficient to cause cell death and some cell cycle-associated events are required for their toxicity.

The c-*myc* oncogene is implicated in cellular proliferation, differentiation and apoptotic cell death [12, 13, 27]. The regulation of c-*myc* expression is extremely complex. Three distinct promoters (P0, P1 and P2) have been characterized but the modulation of c-*myc* transcription also involves a variety of *cis*- and *trans*-acting element located in the 5′ region of the gene [12].

Despite the significance of c-*myc* for both cellular proliferation and apoptotic cell death, little is known about the modulation of the expression of the gene by anticancer agents. Since DNA is the critical target for platinum-induced cytotoxicity, it was interesting to investigate the effect of *cis*-platin and two other platinum compounds (D19466 and D17872) on the expression of c-*myc* promoter by introducing a model consisting of three deletions of c-*myc* promoter region linked to the reporter *cat* gene. It was also interesting to compare such an effect with cytotoxicity data obtained by using the MTT assay and a clonogenic capillary microassay.

We have found that *cis*-platin and D19466 (lobaplatin) induce a significant increase in c-*myc* promoter-driven *cat*-activity in the MEL cell line F412B2TK$^-$ in a dose and time-dependent manner, showing the maximum of their effect after 24 hr exposure to the cytotoxic concentration of 5×10^{-5} M (increased expression of 7.4- and 8.1-fold, respectively). On the other hand, the less cytotoxic compound D17872 only slightly activates the c-*myc* promoter at concentrations of 5×10^{-5}–1×10^{-4} M and has no effect at lower concentrations. Induction of the c-*myc* promoter after platinum treatment was

also observed in Rat-1 fibroblasts and the human erythroleukaemic cell line K562 transfected with the same *myc*-promoter/*cat* constructs which underlies the generality of the phenomenon. On the contrary, no significant increase in the c-H*ras*1 promoter-driven *cat*-activity was found when the F122A-2 cell line was treated with a variety of concentrations of the three platinum analogues, suggesting that their effect is selective to specific gene promoters.

The inducing signal for c-*myc* is possibly a direct consequence of DNA platinum binding. DNA damaging agents such as UV radiation and alkylating drugs have been reported to cause subtle changes in chromatin structure correlated with increased levels of c-*myc* expression [17]. Selective damage of the c-*myc* promoter has been found in small cell lung carcinoma cell lines treated with topoisomerase II inhibitors such as amsacrine and etoposide [30]. Furthermore, Futscher *et al.* [28] detected nitrogen mustard induced DNA crosslinks in the over-expressed c-*myc* but not in the weakly expressed N-*ras* gene in a Colo320 cell line treated with high drug concentrations, suggesting that DNA damage is produced and processed in the genome in a non-random fashion. Similar results were previously described by Jones *et al.* [18].

Taking into account these data together with our observations, we propose that DNA damage induced by platinum drugs may selectively affect the expression of certain genes, such as the c-*myc* proto-oncogene. Overexpression of c-*myc* has been correlated with apoptotic cell death [13] and could therefore be a potential contributor to the cytotoxic outcome. It is possible that the observed c-*myc* induction in high but not low *cis*-platin and D19466 concentrations is due to or related to the formation of a critical number of DNA–platinum adducts in the c-*myc* promoter. The virtual lack of response of the c-*myc* promoter to the less cytotoxic D17872 could support such a hypothesis.

It is further interesting that exposure of cells to these three platinum compounds revealed a difference in the expression of the c-*myc* deletions −350 to +511 and −290 to +511 relative to c-*myc* transcription start site P1 (plasmids p330/p323, cell lines F330-3/F323-1, respectively, Fig. 1b). Although there is only a 60 bp difference between them, the −290 to +511 deletion presents a statistically significant difference in *cat*-activity when compared with that of −350 to +511 deletion (Fig. 3). The −290 to −350 region contains a negative regulatory element that binds the transcription factor FOS/JUN (AP-1) [29] which has been shown to be important for c-*myc* down modulation during the HMBA-induced differentiation of Friend cells [10]. Moreover, the region −2300 to −350 (plasmids p324/p330), which strongly responds to platinum complexes, may contain regulatory elements crucial for modulation of c-*myc* expression after drug exposure.

We will test these hypotheses by attempting to identify the site(s) of *cis*-platin binding to the c-*myc* promoter using an *in vivo* adduct identification method based on polymerase chain reaction. Furthermore we will test the possibility of changes in the levels of *trans*-acting factors being responsible for the upregulation of c-*myc* after platinum exposure.

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