INHIBITION OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE BY CISPLATIN AND ITS ROLE IN DRUG ACTION

By

Christine S.M. Chiu

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BY

CHRISTINE S.M. CHIU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACTS

Cis-dichlorodiammine platinum (II) or cisplatin (CDDP) is a widely used antitumor agent in the treatment of a number of malignancies. It is generally believed that the cytotoxic properties of CDDP are most likely a consequence of interaction with its major intracellular target DNA, resulting in inhibition of DNA synthesis and cell division. However, detailed binding studies of CDDP to DNA strongly suggested that this can not adequately explain the cytotoxic effect of the drug. Recently, ribonucleotide reductase from *E.coli* has been shown to be potently inhibited by CDDP suggesting that ribonucleotide reductase may be a critical site of action for CDDP. It is worthwhile to investigate the possible involvement of mammalian ribonucleotide reductase in the cytotoxic action of the drug, since mammalian ribonucleotide reductase is a highly cell cycle regulated enzyme, which converts ribonucleotide diphosphates to deoxyribonucleotide diphosphates, and therefore it plays an important role in the regulation of DNA synthesis. In this study, we have discovered that mammalian ribonucleotide reductase is inhibited by CDDP. The mechanism of enzyme inhibition involves drug interaction with both M1 and M2 subunits of mammalian ribonucleotide reductase, and perhaps through interaction of the bifunctional coordination of platinum to the nucleophilic sulfhydryl groups on both subunits. Studies on the cytotoxic effects of CDDP on hydroxyurea-resistant mouse cell lines suggest that mammalian ribonucleotide reductase is unlikely to be a primary site of drug action *in vivo*. Nevertheless, we conclude that CDDP inhibition

of mammalian ribonucleotide reductase may play a secondary role in the lethal activity of the drug in cancer chemotherapy.

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Finally, I would like to thank my parents and my husband for their support and understanding during the completion of this thesis.

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ABBREVIATIONS

α-MEM	Alpha modified minimal essential medium
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatases
BSA	Bovine serum albumin
CDDP	Cis-dichlorodiammineplatinum(II) or Cisplatin
CDP	Cytidine 5'-triphosphate
cpm	Counts per minute
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
dGDP	2'-Deoxyguanosine 5'-diphosphate
dGTP	2'-Deoxyguanosine 5'-triphosphate
dTMP	2'-Deoxythymidine 5'-monophosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
dUTP	2'-Deoxyuridine 5'-triphosphate
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid disodium
FBS	Fetal bovine serum

GITC	Guanidinium isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IgG	Immunoglobulin G
KD	Kilodalton
mRNA	Messenger ribonucleic acid
MW	Molecular weight
R.Rase	Ribonucleotide reductase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulfonyl fluoride
Pt	Platinum
RPE	Relative plating efficiency
SH	Sulfhydryl
SDS	Sodium dodecyl sulfate
Tris-HCL	Tris(hydroxymethyl)aminomethane hydrochloride
UDP	Uridine 5'-diphosphate

INTRODUCTION

1. CISPLATIN

1.1. Discovery of Cisplatin as an Anticancer Drug

Cisplatin or CDDP (cis-diamminedichloroplatinum II) is the first member of a new class of potent anticancer drugs, the metal coordination complexes, to enter general use in cancer chemotherapy. The biological properties of the simple platinum coordination complexes were discovered serendipitously by Rosenberg and his colleagues (Rosenberg *et al.*, 1965, 1967). The first signal of biological activity occurred when *E.coli* was incubated in a chemically defined growth medium containing ammonium chloride as a nitrogen source, in a chamber containing two platinum electrodes. It was noted that the density of bacteria in the chamber decreased with time when an alternating voltage was applied across the electrodes, but the growth resumed when the voltage was turned off. It was also found that with the field on, the bacteria transformed into long filamentous structures. Later on, they discovered it was a small amount(~ 10ppm) of an electrolysis product of the platinum electrode formed in the presence of the ammonium chloride in the nutrient medium, which caused the filamentation of bacteria. Further analysis showed that this chemical was the classic Peyrone's Chloride, cis-dichloroammine-platinum (II), or its higher oxidation state equivalent, cis-tetrachlorodiammineplatinum(IV) (Rosenberg *et al.*, 1967). Hereafter. the Peyrone's Chloride was referred to by its drug generic name, cisplatin. They also studied other effects of platinum complexes on bacteria and found that charged species in solution were potent bacteriocides, whereas the neutral

species (such as CDDP) inhibited cell division without marked effects on the growth rate, thus leading to filamentation as well as lytic phenomena. In 1968, near the termination of their studies they made the intuitive jump to the idea that this action in bacterial cells, may also inhibit cell division in rapidly growing cancer cells. The first tumor system used for testing these interesting complexes for anticancer activity was the solid sarcoma 180 in mice. The development of this tumor was completely inhibited by CDDP (Rosenberg *et al.*, 1969, 1970). Within the next year, additional reports of clinical trials by various oncology study groups, supported by the NCI in U.S.A., appeared in the literature. In 1971-72, the NCI and the Wadley Institutes of Molecular Medicine both established preclinical pharmacology and toxicology tests, and then introduced the drug into clinical phase I trials. FDA (Federal Department of Agriculture, U.S.A.) approval for the compound came in 1979, and in 1984 it was one of the most widely sold anticancer drugs in the United States.

1.2. Clinical Aspects of Cisplatin

CDDP has been shown to be an effective antineoplastic agent in the treatment of a variety of tumors, such as testicular, ovarian, head, neck, bladder, prostate and lung cancers (Loehrer *et al.*, 1984). CDDP has also been used in combination with other drugs such as bleomycin, VP16, DVA, cytoxan, adriamycin, methotrexate, etc. (Burchenal *et al.*, 1980). Most patient tumors are exposed to CDDP at serum concentrations of 1 to 5 μM with a pharmacological half-life (in patients) of 15-45 min (Rosenberg, 1985). Many clinical trials are prevalent throughout the world,

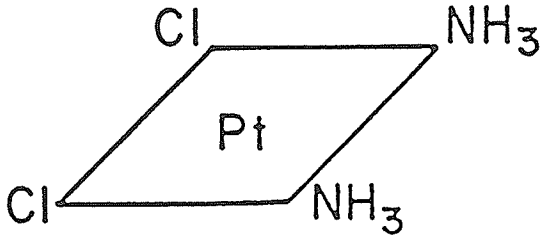
testing both the application of this drug to a variety of cancers, and the improved protocols with higher activity against the known responsive cancers.

However the toxic side effects of CDDP therapy are considerable, including nephrotoxicity, gastrointestinal toxicity, neurotoxicity, ototoxicity (tinnitus and hearing loss), and visual disturbances (Prestayko *et al.*, 1979; Stephens *et al.*, 1979; Krakoff, 1979; Rossof *et al.*, 1972; and Medias & Harrington, 1978). Of these, renal impairment and neurotoxicity are the major dose-limiting toxicities. Intravenous hydration with mannitol diuresis and hypertonic saline (Ozols *et al.*, 1984) and administration of sulfur-containing platinum-binding agents, such as penicillamine (Higby *et al.*, 1975; Slater *et al.*, 1977), thiourea (Burchenal *et al.*, 1979), and sodium thiosulfate (Howell *et al.*, 1982; and Borch *et al.*, 1979.), have been used to reduce the severity of nephrotoxicity. CDDP-induced myelosuppression has also been noted in several clinical studies, although not dose-limiting (Rossof *et al.*, 1972; Medias & Harrington, 1978; and Einhorn *et al.*, 1979).

1.3. Aqueous Chemistry of Cisplatin

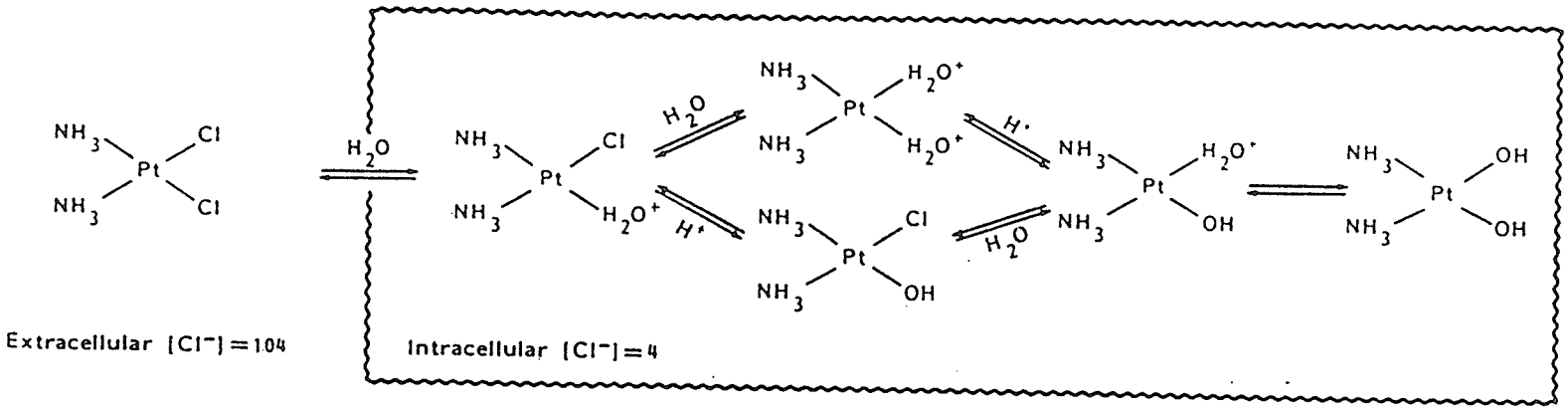
CDDP is a neutral, water-soluble square planar coordination complex containing a central platinum atom surrounded by two chloride atoms and two ammonia moieties (Figure 1). The antitumor activity of the complex is much greater when the chloride and ammonia moieties are in the cis position compared to the trans position. In aqueous solution, it undergoes hydrolysis to form a variety of partially and fully hydrolysed species (Figure 2) (Howe-Grant & Lippard, 1980). An

Figure 1. Chemical structure of cisplatin (Harder & Rosenberg, 1970).



Cis-Platinum (II)
Diamminodichloride

Figure 2. The hydrolysis reaction of cisplatin (Loehrer *et al.*, 1984).


Extracellular [Cl⁻] = 104
Intracellular [Cl⁻] = 4

additional driving force for substitution of chloride by water is loss of a proton from the aquo species to form various hydroxy species. Throughout these equilibria, the cis configuration is retained. Hydroxy-bridged dimers, trimers and higher oligomers are also formed in all but dilute solution (Lim & Martin, 1976; and Faggiani *et al.*, 1977).

CDDP is administered to patients as an intravenous injection. The concentration of chloride ion in plasma (103 mM) is sufficiently high to maintain CDDP in the neutral, dichloro form. After entering across the lipid membrane, however, the chloride ion concentration decreases sharply to 4mM, promoting the hydrolysis of the labile chloride ligands. From thermodynamic data on chlorodiethlenetriamineplatinum(II), it has been estimated that the relative concentration of the various aquated species in plasma is 2% of total platinum and that the drug enters the cytoplasm (Lim & Martin, 1976). The aquated species subsequently reacts with a variety of intracellular components.

The mechanism whereby CDDP enters cells remains an enigma (Andrew & Howell, 1990). It has long been assumed that CDDP enters by passive diffusion, but a large body of evidence now indicates that accumulation of the drug can be modulated by means that seem incompatible with only simple passive uptake because cisplatin accumulation is: (1) energy dependent, Na^+ dependent, and ouabain inhibitable (Andrew *et al.*, 1988a, 1988b); (2) stimulated by reductions in osmotic strength and pH (Andrew *et al.*, 1988b; and Smith & Brock, 1989); (3) inversely related to membrane potential; (4) stimulated by elevation of cAMP levels; and (5)

inhibited by aldehydes (Dornish & Peterson, 1989). In spite of this evidence, classical proof for a carrier system is lacking in that accumulation of the drug is not saturable up to 3mM CDDP, and cannot be inhibited with structural analogs (Andrew *et al.*, 1988b; and Mann *et al.*, 1990). There is one exception however, since active transport of CDDP by an amino acid transport system has been shown in mitogen-stimulated lymphocytes (Byfield & Calabro-Jones, 1981).

1.4. Possible Mechanisms for the Antitumor Activity of Cisplatin

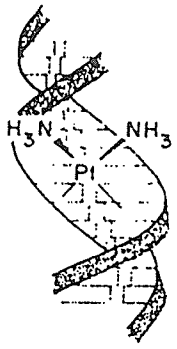
The antitumor activity of the drug is generally believed to result from its interaction with DNA (Roberts & Thomson, 1979; Sherman & Lippard, 1987). It was found that CDDP selectively and persistently inhibited the rate of DNA synthesis as compared with effects on RNA and protein synthesis in human AV3 cells (Harder & Rosenberg, 1970) and Hela cells (van den Berg *et al.*, 1977) in culture, and in Ehrlich ascites cells (Howle & Gale, 1970) *in vivo*. Selective inhibition of DNA synthesis has also been demonstrated in phytohemagglutin-stimulated human peripheral lymphocytes (Howle *et al.*, 1971), in the folate-stimulated kidney, and in the intestinal mucosa of normal and tumor-bearing rats. Rous Sarcoma virus-stimulated DNA synthesis in infected chick embryo cells was also blocked irreversibly by CDDP and it also inhibited subsequent cellular transformation. It is now clear that CDDP bound to primed templates inactivates them as substrate for human DNA polymerase α and β as well as for reverse transcriptase, but does not affect transcription or translation. It is still unknown what kind of CDDP-DNA interaction,

i.e., which CDDP adduct, is responsible for this action.

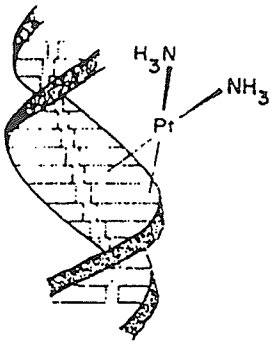
Many different techniques have been developed over the years to establish the identity and relative occurrence of the various adducts formed in DNA (Pinto & Lippard, 1985; Fichtinger-Schepman *et al.*, 1982, 1985a, 1985b, 1987; Eastman, 1986; and Johnson, *et al.*, 1985). Several types of interactions have been proposed: DNA interstrand crosslinks, DNA intrastrand crosslinks and crosslinks between DNA and proteins (Figure 3).

Interstrand Cross-Linking: various quantitative methods have been designed to estimate CDDP-induced DNA interstrand crosslinks (Robert & Friedlos, 1987) and most studies have used the alkaline elution method (Fram *et al.*, 1987). There have been numerous efforts to correlate interstrand crosslinking with cytotoxicity. However, the results are inconsistent. Some cell lines exhibited a positive correlation (Zwelling *et al.*, 1979, 1981; and Laurent *et al.*, 1981), but a significant number of others did not (Zwelling *et al.*, 1981; Laurent et al., 1981; and Strandberg *et al.*, 1982). Indeed, there was considerable evidence that the interstrand crosslink was unlikely to be a critical lesion responsible for the cytotoxicity of CDDP. It was estimated that DNA interstrand cross-links formed in mammalian cells constitute less than 1% of total platinum(Pt)-DNA adducts shortly after treatment (Ploody *et al.*, 1984). Statistically, this percentage is too low to account for the cytotoxicity of CDDP (Shooter *et al.*, 1972; Munchausen, 1974). Furthermore, study of the inactivation of the double-stranded DNA phage T7 by dichloroethylenediamineplatinum(II), a compound closely related to CDDP structurally and having significant antitumor activity, revealed that

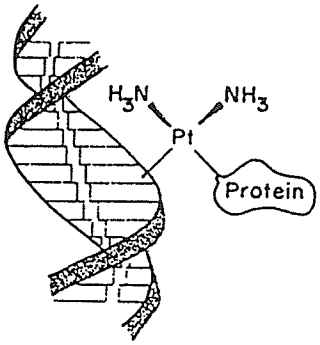
Figure 3. Possible bifunctional binding modes of CDDP with DNA (Sherman & Lippard, 1987).



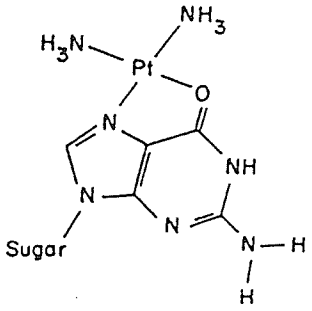
Interstrand Crosslink



Intrastrand Crosslink



DNA - Protein Crosslink



Bifunctional Binding to Guanine

as few as five platinum atoms bound per phage were needed to destroy its biological activity (Shooter *et al.*, 1972). However, 30 atoms per phage were required for introducing one interstrand crosslink into the phage DNA.

DNA-Protein Cross-Linking: The alkaline elution method has also been applied to measure the amount of DNA-protein crosslinks. It was found that they comprised only a very small fraction (0.15%) of the total Pt-DNA adducts formed *in vivo* (Plooy *et al.*, 1984) and this did not correlate well with cytotoxicity (Zwelling *et al.*, 1979; and Laurent *et al.*, 1981).

Intrastrand Cross-Linking : biochemical studies using restriction endonucleases have been applied to study intrastrand crosslinks (Cohen *et al.*, 1980; Ushay *et al.*, 1982; Tullius & Lippard, 1981; and Royer-Pokora *et al.*, 1981; Sherman & Lippard, 1987), and have revealed the strong preference of CDDP to bind to guanine-rich regions of DNA. Furthermore, physical studies of short oligonucleotides and their constituents using spectrophotometry (Munchausen & Rahn, 1975) indicated that an intrastrand crosslinking between the N7 atoms of the adjacent guanines was the principle adduct formed between CDDP and DNA. The preference of CDDP for the N7 atom of guanine is due to the high electron density at N7 which makes it susceptible to attack by electrophilic metal ions; and N7 lies in the major groove of B form DNA and is thus readily accessible to incoming reagents. The charge and cis configuration of the $[cis\text{-}Pt(NH_3)_2]^{2+}$ fragment facilitates chelation to N7 atoms of adjacent guanines. Using anion-exchange chromatography to isolate and characterize DNA adducts, it was found that the principle adduct was composed of

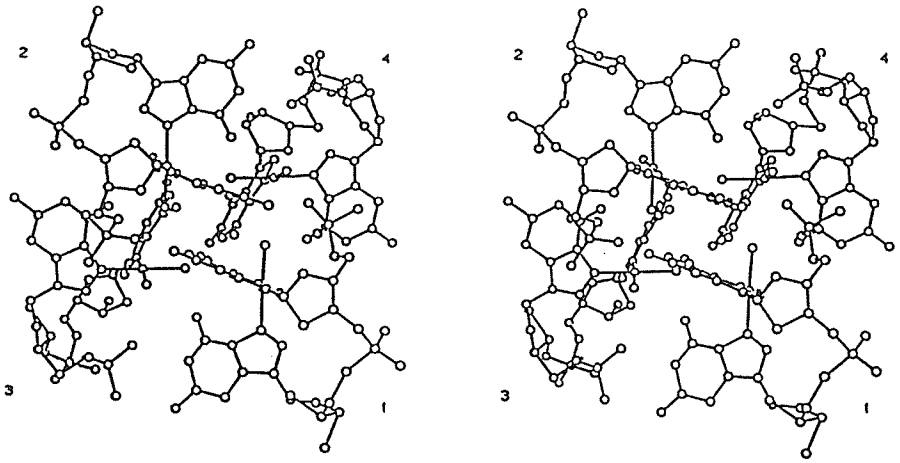
intrastrand crosslinking of two adjacent guanines, cis-[pt(NH_3)$_2$d(pGpG)] (Fichtinger-Schepman, *et al.*, 1984). Crystals of the adduct of CDDP with d(pGpG) had also been obtained and used in an X-ray crystallographic study to elucidate the molecular structure to atomic resolution (Figure 4a,b) (Sherman *et al.*, 1985). *In vivo* study of intrastrand crosslinking was achieved by applying immunological techniques (Poirier *et al.*, 1982; and Lippard *et al.*, 1983). They also demonstrated that the d(GpG) crosslink was the major adduct.

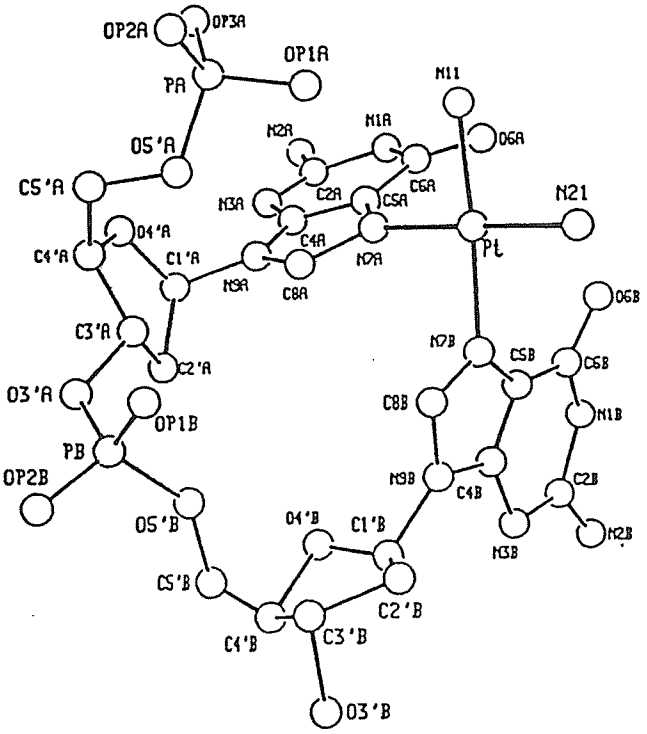
Interactions with other intercellular molecules: although it is generally believed that interactions of CDDP with chromosomal DNA are responsible for the antitumor activity of CDDP, membrane and cytosolic components also affect the biological activity of CDDP.

A number of reports suggested that inhibition of cellular enzymes by CDDP may also play an important role in its cytotoxic action. Particularly, enzymes which contain reactive sulfhydryl (SH) groups were extremely sensitive to CDDP. A series of dehydrogenase enzymes (nicotinamide dehydrogenase, malate dehydrogenase, lactate dehydrogenase and alcohol dehydrogenase) was inhibited by CDDP (Teggins & Friedman, 1974), and all have catalytically important SH groups. Glyceraldehyde-3-phosphate dehydrogenase containing an essential SH group was readily inhibited by CDDP (Aull *et al.*, 1979). More recently, ribonucleotide reductase from *E.coli* was stereoselectively and strongly inhibited by CDDP (Smith & Douglas, 1989). Some studies suggested the general mechanism of nephrotoxicity was due to the binding of the platinum to SH groups in the proximal tubule with possible inhibition of enzymes

Figure 4a. Stereo view of the four crystallographically independent *cis*-[$Pt(NH_3)_2${d(pGpG)}] molecules in the unit cell. The view is down the *a* axis of the unit cell, revealing the pseudo-twofold symmetry of the aggregate. Water and glycine molecules in the lattice are not shown (Sherman & Lippard, 1985).

Figure 4b. Molecular structure of one of the four *cis*-[$Pt(NH_3)_2${d(pGpG)}] molecules portraying the atom labelling scheme (Sherman & Lippard, 1985).


2
4
3
1
2
4
3
1


OP2A
OP3A
OP1A
PA
O5'A
C5'A
C4'A
O4'A
C1'A
C3'A
C2'A
O3'A
OP1B
PB
OP2B
O5'B
C5'B
C4'B
C3'B
O3'B
C2'B
O4'B
C1'B
N2A
N1A
O6A
C2A
C6A
N3A
C5A
N21
C4A
N7A
Pt
N9A
C8A
N11
O6B
N7B
C5B
C6B
C8B
N1B
N9B
C4B
C2B
N3B
N2B

and alterations of membrane permeability (Levi *et al.*, 1980; Borch & Pleasants, 1979; Weiner & Jacob, 1984; and McCarley & Dennis, 1982). The results of the enzyme histochemical studies suggested that acid phosphatase of lysosome in the proximal tubule was inhibited by CDDP, which might result in lysosomal accumulation (Jones *et al.*, 1985). CDDP was found to inhibit several types of *in vitro* renal ATPase activities activated by $Na^+ + K^+ + Mg^{2+}$, Mg^{2+} or Ca^{2+} (Nechay & Neldon, 1984). The fact that platinum accumulated in the kidneys of animals treated with CDDP led to the suggestion that under certain circumstances ATPase activities may be inhibited *in vivo* (Weiner & Jacob, 1984).

As suggested before, the primary mechanism by which CDDP prevents tumor growth appears to involve binding of the drug to DNA and inhibition of nucleotide synthesis. However such a conclusion does not adequately explain a number of other "facts", particularly the disappearance of large solid tumors in many systems following treatment with dose levels apparently not cytotoxic to the tumor. Nor does such a mechanism of action explain the apparent selective killing of certain tumor cells. Earlier, Rosenberg suggested that activation of host defense mechanisms may participate in platinum-mediated tumor cytoreduction (Rosenberg, 1975). In agreement with this suggestion, Kleinerman *et al.* (1980a), have demonstrated that *in vitro* exposure to CDDP resulted in an enhancement of spontaneous monocyte-mediated cytotoxicity. Also, cancer patients treated with CDDP demonstrated a significant rise in spontaneous monocyte mediated cytotoxicity over their pre-treatment values (Kleinerman *et al.*, 1980b). More recently, Lichtenstein and Pende

(1986) demonstrated that CDDP was a potent activator of natural killer (NK) effector function in mice, and that it increased the sensitivity of tumor targets to effector cell lysis.

1.5. Possible Mechanisms of Acquired Resistance to Cisplatin

A tremendous effort has been put into elucidating the mechanisms by which cancer cells acquire their resistance to CDDP (Rosenberg, 1985; and Andrew & Howell, 1990). After an initially successful response towards CDDP treatment, some tumor cells rapidly develop resistance (Rosenberg, 1985; Ozols *et al.*, 1984; Scanlon & Kashani, 1989c; and Zwelling, 1987). Until now, studies on the mechanism of acquired resistance to CDDP were all performed with cultured cells; several possibilities have been suggested to explain the phenomenon. In most cases the decreased sensitivity of the cells to CDDP was ascribed to a combination of the following factors noted below.

Decreased cisplatin accumulation: Decreased CDDP accumulation has been a consistent finding in many CDDP-resistant cell lines from a variety of species (Andrew *et al.*, 1990). A reduction in CDDP influx was suggested to be at least partly involved in the resistance phenotype since decreases in accumulation could be detected as early as 30 sec after the start of drug exposure (Mann *et al.*, 1990; Waud, 1987; and Hromas *et al.*, 1987). CDDP-resistant 2008 cells (human ovarian carcinoma cells in athymic mice) had the same membrane fluidity as sensitive cells, although some changes in the cellular phospholipid composition were found (Mann *et al.*,

1988). Timmer-Bosscha *et al.* (1989) also found changes in the lipid composition of CDDP-sensitive versus CDDP-resistant small cell lung carcinoma cells, although the latter cells did not have an accumulation defect. These observations led to the interpretation that CDDP was transported into or out of the cell by a carrier protein that was somehow changed in the resistant cells.

Elevation of intracellular protein and non-protein sulfhydryl molecules: CDDP is an electrophile that is most reactive toward sulfur containing nucleophiles. Glutathione (GSH) obviously is a good candidate and one would thus predict that resistant cells may contain elevated levels of GSH. However, the role of GSH in mediating CDDP resistance is hitherto unclear. Results of current studies indicate a combination of positive, negative, as well as no correlation between cellular GSH level and degree of resistance. Normal human lung fibroblasts, which had a 50% elevation of GSH levels induced with 2-oxothiazolidine-4-carboxylate, showed a 1.4-fold increase in the CDDP resistance (Russo *et al.*, 1986). Hospers *et al.* (1988) found that GSH was elevated 3.4-fold in a CDDP-resistant human small cell lung carcinoma cell line. Hamilton *et al.* (1985) reported that 14-fold CDDP-resistant A2780 human ovarian carcinoma cells had 3.2-fold elevated GSH. Both CDDP-resistant and wild type parent A2780 cells were sensitized to CDDP when GSH depletion was maintained throughout the CDDP exposure. GSH was minimally elevated in CDDP-resistant mouse L1210 and P388 leukemia cells. *In vivo* selection in two different models produced resistant tumors without elevated GSH (Ferrare *et al.*, 1989). Andrew *et al.* (1985) found that 2 to 3-fold CDDP-resistant 2008 cells did not have

elevated levels of GSH. One study showed that CDDP-resistant rat ovarian granulosa cell tumors (0-342/DDP) were obtained with a 1.9-fold increase in GSH levels at the time of sacrifice, but after 24 hr in culture, the 0-342/DDP cells actually had 25% less GSH than the cells from the sensitive tumor (Chen *et al.*, 1989). Elevation of GSH-transferase levels has also been found in some CDDP resistant tumor cells (Andrew *et al.*, 1990), and its role in CDDP resistance is unknown at present. Interestingly, one report suggested that low levels of the GSH-transferase pi enzyme in small cell lung cancer cells correlated with CDDP sensitivity (Nakagawa *et al.*, 1988).

Therefore, there is no direct and consistent evidence that GSH inactivates enough intracellular CDDP to affect CDDP cytotoxicity, and it has been asked whether the rise of GSH is just simply part of a general stress response to CDDP (Andrew *et al.*, 1990).

Metallothioneins (MTs) comprise a family of MW 6000-7000 proteins involved in Zn^{2+} homeostasis and in the detoxification of heavy metals (Hamer, 1986). MTs are composed of 30% cysteine and can account for a large percentage of the intracellular thiol content. Since they are rich in thiol groups, MTs are likely targets for electrophilic agents such as CDDP. Numerous studies have shown that MTs can bind CDDP in normal as well as in tumor tissues. In both rat liver and kidneys, 25% of the total cytosolic Pt was reported to be bound to MT-like proteins following CDDP treatment (Zelazowski *et al.*, 1984; Mason *et al.*, 1984; and Sharman & Edwards, 1983). Cd^{2+} -selected cells, which contained elevated MTs, were cross-resistant to CDDP (Bakka *et al.*, 1981; and Andrew *et al.*, 1987). Mouse mammary

C127 cells, which had a 10-fold increase in MT content by transfecting with a human MT-IIA gene were 4-fold resistant to CDDP (Kelley *et al.*, 1988). Also, a variety of CDDP resistant cells were shown to have elevated MTs (Kelley *et al.*, 1988). However, Schelder *et al.* (1989) reported that elevation of MTs did not correlate with CDDP resistance. Eastman *et al* (1988a) showed that highly CDDP resistant L1210 cells did not have MTs elevation and were hypersensitive to Cd^{2+}. It is apparent that overexpression of MTs does confer CDDP resistance, and that these proteins do bind intracellular CDDP. However, what is not clear is whether elevation of MTs is an important mechanism of resistance in cells subjected to clinically relevant selection procedures.

Increased repair of drug-induced intrastrand DNA crosslinks: Studies up to now tend to demonstrate that DNA is the major cellular target for CDDP. Increased DNA repair capacity has been suggested as a mechanism for CDDP resistance. Studies have shown that cell lines defective in DNA repair were indeed hypersensitive to CDDP cytotoxicity (Eastman *et al.*, 1988a; and de Graeff *et al.*, 1988). CDDP-resistant A2780 cells have enhanced DNA repair capacity as indicated by increased unscheduled DNA synthesis and repair synthesis when exposed to CDDP (Ozols *et al.*, 1988; and Lai *et al.*, 1988). CDDP resistant L1210 cells also removed the dGpdG intrastrand cross-link more rapidly than did sensitive cells (Eastman & Schulte, 1988b). Some enzymes involved in DNA synthesis and DNA repair have also been shown to be elevated in CDDP resistant cells. DNA polymerase ß activity was elevated in CDDP resistant P388 leukemia cells, and these cells also exhibited

increased unscheduled DNA synthesis (Kraker & Moore, 1987 & 1988). Scanlon *et al.* have shown that polymerase α and ß mRNA and enzyme activity were elevated in CDDP resistant HCT8 human colon carcinoma cells and A2780 human ovarian carcinoma cells, that were generated by pulsing cells with CDDP for 1 hr once per week (Scanlon *et al.*, 1989a & 1989b). Attempts have been made to modulate CDDP cytotoxicity by inhibiting various DNA repair processes. For example, aphidicolin, an inhibitor of DNA polymerase α, has been shown to potentiate CDDP cytotoxicity in CDDP resistant but not CDDP sensitive A2780 cells (Ozols *et al.*, 1988). Adenosine-ribose transferase (ADPRT) is another enzyme believed to have important functions in DNA repair. Inhibition of ADPRT with 3-aminobenzamide or nicotinamide, sensitized Erhlich ascites and sarcoma 180 tumors to CDDP in mice (Chen & Pan, 1988). 3-aminobenzamide, however, did not affect the cytotoxicity of CDDP toward cultured ovarian carcinoma cells (Umbach *et al.*, 1985). 1-ß-D-arabinofuranosylcytosine and hydroxyurea together inhibited excision repair, and this combination was synergistic with CDDP in the HT-29 human colon carcinoma cell line (Swinnen *et al.*, 1989).

Altered Folate Metabolism: Some CDDP resistant cell lines exhibit changes in folate metabolism. A2780 human ovarian carcinoma cells that were made 3.2-fold resistant by pulsing weekly with 50μM CDDP for 1 hr showed 2.5-fold increases in thymidine kinase, thymidylate synthase, and dihydrofolate reductase activities (Scanlon *et al.*, 1989b). Similar changes were found with HCT8 human colon carcinoma cells (Scanlon *et al.*, 1989a). Since CDDP can inhibit methionine transport,

Scanlon *et al.* (1989b) hypothesized that this inhibition perturbed methionine/1-carbon metabolism, which in turn stimulated folate metabolism. The stimulated folate metabolism might be one of the first changes to occur during development of resistance, since it was found in cells with low-level CDDP resistance. 12-fold CDDP resistant SCC25 human head and neck carcinoma cells that were selected by weekly pulses with escalating concentrations of CDDP, were 8-fold cross resistant to methotrexate suggesting that these cells also have altered folate metabolism (Rosowsky *et al.*, 1987). Elevated levels of enzymes in the dTMP cycle may be needed for enhanced DNA repair since this cycle provides dTMP.

Role of oncogenes: Sklar (1988) has shown that NIH-3T3 cells transformed with *ras* oncogenes activated by missense mutations or by overexpression of the normal c-H-*ras* gene become 2 to 8.5-fold CDDP resistant (Sklar, 1988). Transformation of these cells by overexpression of v-*fms* or v-*mos* also conferred 2-fold CDDP resistance. Scanlon *et al.* (1989b) and Sklar (1988) have reported that CDDP resistant A2780 cells express higher levels of *ras*, *fos* and *myc* mRNA. Similar results were found with CDDP resistant HCT8 cells except that *myc* mRNA levels were unchanged. Human ovarian carcinoma tissue from patients, who have failed CDDP-based chemotherapy, has been reported to contain amplified *fos* DNA and elevated levels of *fos* and *ras* mRNA (Scanlon *et al.*, 1989b; and Sklar, 1988). There is as yet no biochemical explanation for the effects of *ras*, *fos*, or *myc* on the cytotoxicity of CDDP.

Which of these biochemical changes eventually predominates may depend upon the type of cell, and particularly on the variant selection procedure. Chronic, long-term exposure to increasing concentrations of CDDP seems to lead to permanent elevations in the levels of the nucleophiles GSH and MTs. Weekly, pulsed exposures lead to changes in folate metabolism and oncogene expression, and monthly, acute exposures lead to defects in drug accumulation. In addition, which of these selection procedures are most relevant to the development of CDDP resistance in a patient's tumor is not obvious yet.

2. MAMMALIAN RIBONUCLEOTIDE REDUCTASE

2.1. A Brief Introduction to Mammalian Ribonucleotide Reductase

Mammalian ribonucleotide reductase is a highly regulated enzyme, which is responsible for the *de novo* reduction of ribonucleotides to their corresponding deoxyribonucleotides, the precursors of DNA synthesis (Wright *et al.,* 1990; Wright, 1989a). Since, this reduction reaction is a rate-limiting step in DNA synthesis, the enzyme plays an important role in the regulation of cell division, and hence cell proliferation.

The reduction reaction is summarized in Figure 6 (Wright, 1989a). Two small proteins, thioredoxin through the thioredoxin reductase system and glutaredoxin via glutathione and glutathionine reductase (Wright, 1983; 1989a; Wright *et al.*, 1990) can function as hydrogen carriers in the reaction.

2.2. Structure of Mammalian Ribonucleotide Reductase

Like the enzyme from *E.coli*, the mammalian ribonucleotide reductase consists of two nonidentical subunits that are often referred to as M1 and M2 (Figure 5), both of which have been purified to homogeneity (Thelander *et al.*, 1980, 1985). The M1 protein is a dimer of MW 170,000, and it contains the binding sites for substrates and nucleoside triphosphates which act as allosteric effectors (Thelander *et al.*, 1980). The M2 protein is a dimer of MW 88,000, and it contains a non-heme iron and a tyrosyl free radical essential for activity (McClarty *et al.*, 1990). Enzyme activity depends

Figure 5. Mammalian ribonucleotide reductase model showing the M1 and M2 components. Note the M1 activity (O) and substrate specificity (◇) sites, and the M2 nonheme iron and tyrosyl-free radical (Wright *et al.*, 1990).

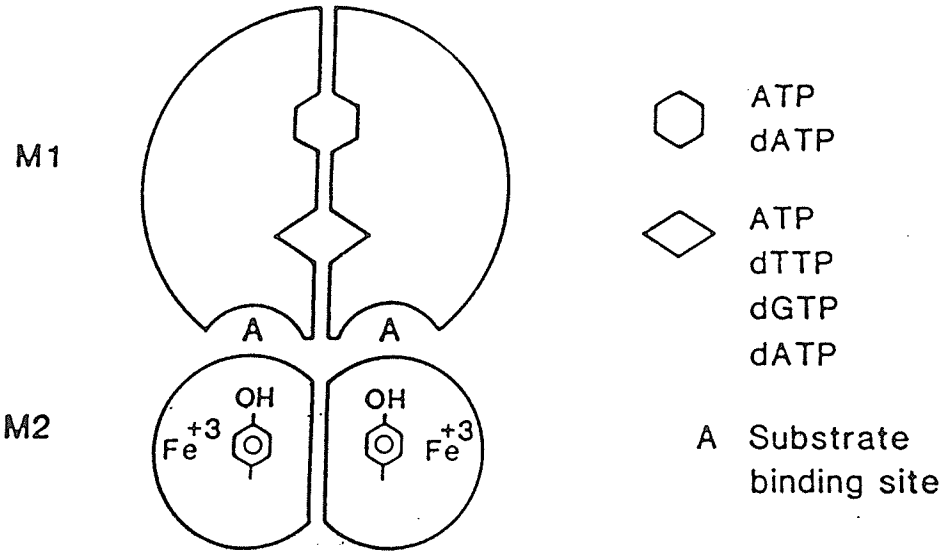
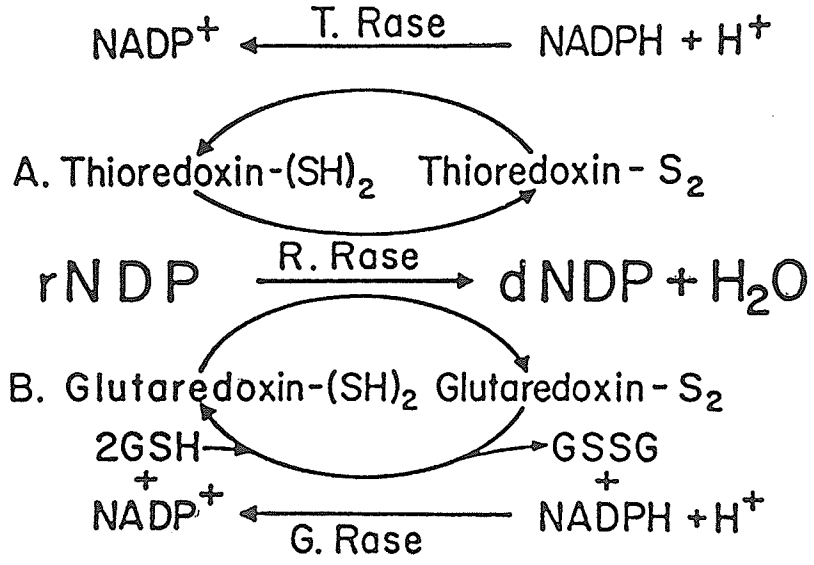

M1
M2
A
A
OH
OH
Fe^{+3}
Fe^{+3}
ATP
dATP
ATP
dTTP
dGTP
dATP
A Substrate binding site

Figure 6. Reduction of ribonucleotides (rNDP) to deoxy-ribonucleotides (dNDP) by ribonucleoside diphosphate reductase (R.Rase), in the presence of (A) thioredoxin reductase (T.Rase) and thioredoxin, and (B) glutathione reductase (G.Rase), glutathione (GSH) and glutaredoxin (Wright, 1983).

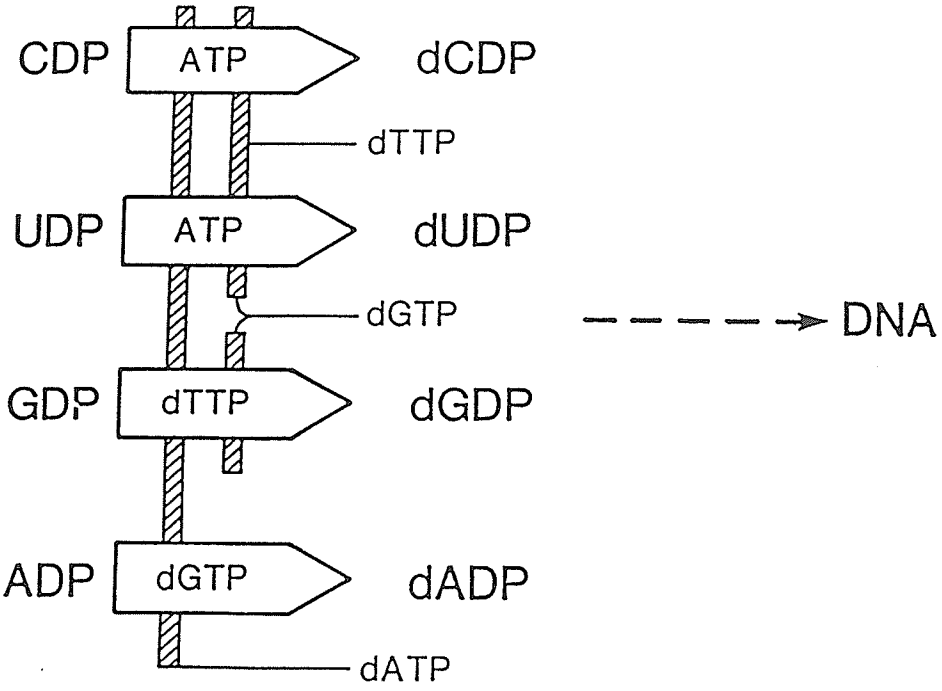

NADP+
T. Rase
NADPH + H+
A. Thioredoxin-(SH)2
Thioredoxin-S2
rNDP
R. Rase
dNDP + H2O
B. Glutaredoxin-(SH)2
Glutaredoxin-S2
2GSH
GSSG
NADP+
G. Rase
NADPH + H+

upon the presence of both M1 and M2 components (Cory *et al.*, 1978; McClarty *et al.*, 1986a). However, different mechanisms control the levels of the two proteins during cell growth (Eriksson and Martin, 1981b; Cory and Fleischer, 1982; Eriksson *et al.*, 1984; Engstrom *et al.*, 1985; Wright *et al.*, 1987; McClarty *et al.*, 1987a; Choy *et al.*, 1988; Wright 1989a; Hurta & Wright, 1990). The M1 subunit appears to be nearly constant throughout the cell cycle of proliferating cells with a half life of 12.5 to 15 hr (Engstrom *et al.*, 1985; Choy *et al.*, 1988), whereas *de novo* synthesis of M2 correlates with S phase and the M2 subunit has a half life of 3 to 6.3 hr (Lewis *et al.*, 1978c; Eriksson *et al.*, 1984; Choy *et al.*, 1988). These suggest that, at least in the cultured cell lines, the M2 protein is limiting for ribonucleotide reductase activity during the cell cycle. However, these regulatory differences are unlike the observation obtained with *E.coli*, where the two equivalent genes (B1 and B2) are located in one operon, and their synthesis is coordinately regulated (Hanke & Fuchs, 1983; Carlson *et al.*, 1984). Also, in contrast to the *E.coli* finding, the rodent and human M1 and M2 genes have been mapped to different chromosomes (Yang-Feng *et al.*, 1987; Tonin *et al.*, 1987) and there appear to be pseudogenes for M2 in mammalian cells (Yang-Feng *et al.*, 1987; Wright *et al.*, 1989b).

2.3. Allosteric Regulation of Mammalian Ribonucloetide Reductase

The substrate specificity and activity of the mammalian ribonucleotide reductase is strictly regulated in a complex fashion by nucleoside triphosphate effectors (Figure 7) (Wright, 1983, 1989a). The reduction of CDP to dCDP and UDP

Figure 7. Allosteric regulation of mammalian ribonucleotide reductase. Inhibitory effects are indicated by the bars and the nucleotides shown in the arrows act as positive effectors (Wright *et al* ., 1990).


CDP
ATP
dCDP
dTTP
UDP
ATP
dUDP
dGTP
DNA
GDP
dTTP
dGDP
ADP
dGTP
dADP
dATP

to dUDP takes place in the presence of an ATP activated enzyme. The reduction of GDP to dGDP requires dTTP, while the reduction of ADP to dADP requires dGTP. Reduction of all four ribonucleotide substrates is inhibited by dATP. The allosteric properties of the enzyme suggest that deoxyribonucleoside diphosphate formation begins with a reduction of CDP and UDP by an ATP activated activity, proceeds to GDP reduction via a dTTP regulated activity, and finally reaches ADP reduction by a dGTP activated activity. Accumulation of dATP, for example, in the presence or slowing down of DNA synthesis, turns off the reductase completely, since dATP is a potent inhibitor of all ribonucleotide reductions. In addition, dTTP is a good inhibitor of pyrimidine reduction and dGTP is a negative feedback effector of GDP reduction and inhibits the reduction of pyrimidines. It has also been mentioned that GTP as well as dGTP may be a positive effector in the reduction of ADP (Chang & Cheng, 1979; Wright *et al.*, 1981; and Hards & Wright, 1984a,b), and although high concentrations of dATP potently inhibit ribonucleotide reduction, very low levels may actually stimulate enzyme activity (Lewis *et al.*, 1978). In addition, combinations of several different nucleotides may act in a synergistic fashion to modify enzyme activity (Hards & Wright, 1984a,b).

2.4. Hydroxyurea and Mammalian Ribonucleotide Reductase

Since ribonucleotide reductase plays an important role in cell division, it becomes a favoured target of numerous antitumor drugs (Elford & van't Riet, 1989; Veale *et al.*, 1988; Moore & Hurlbert, 1989). Hydroxyurea has turned out to be one

of the most valuable antitumor agents.

Hydroxyurea enters mammalian cells by a diffusion process (Tagger, *et al.*, 1987), is cytotoxic for proliferating cells (Wright & Lewis, 1974), and specifically inhibits ribonucleotide reductase by destabilizing the iron centre of protein M2 and thereby destroying the tyrosyl-free radical needed for enzyme activity (e.g. McClarty *et al.*, 1990). Hydroxyurea resistant mutant cell lines have been isolated and characterized in attempts to understand the biological functions of ribonucleotide reductase (e.g. McClarty *et al.*, 1987a; Choy *et al.*, 1988; Hurta & Wright, 1990a,b). Mutant lines exhibiting low to high drug resistance characteristics revealed many different mechanisms for modifying the expression of the two components of ribonucleotide reductase. For examples, mutant cells selected for resistance to N-carbamoyloxyurea and cross resistant to low concentrations of hydroxyurea exhibited an increase in M2 message without any changes in M2 gene copy number, or without any detectable changes in M1 gene expression (Wright *et al.*, 1987; Tagger and Wright, 1988). Also, mutant cells selected in relatively high concentrations of hydroxyurea (e.g. 1.5 mM) overproduced ribonucleotide reductase activity, due to an overproduction of the M2 protein, as a result of increased M2 mRNA levels and an amplification of the M2 gene (Choy *et al.*, 1988). In addition to an increase in protein M2, cell lines selected for resistance to very high concentrations of hydroxyurea (e.g. 30 mM) showed elevated protein M1 levels as well, which resulted from an elevation of M1 mRNA as well as M1 gene amplification (Hurta & Wright, 1990a,b). When mutant cells were cultured in the presence of hydroxyurea, M1 and M2 protein levels

were further elevated, and this elevation was not accompanied by increases in the corresponding mRNAs (McClarty *et al.*, 1987a). Furthermore, it was also noted that the level of both subunits of ribonucleotide reductase responded to hydroxyurea in a drug concentration dependent manner. The increases of M1 and M2 levels were not brought about by increasing the M1 and M2 mRNA levels, but by increasing the stabilization of both proteins against degradation and by increasing their rates of biosynthesis (McClarty *et al.*, 1988). These results suggest that in addition to gene amplification and transcription, the ribonucleotide reductase expression can also be regulated by posttranscriptional modifications.

MATERIALS AND METHODS

1. Cell Lines and Culture Conditions

1.1 Culture Conditions

All cells were cultured on the surface of plastic cell culture plates (Lux Scientific Ltd.) with either alpha modified minimal essential medium (α-MEM) (Flow Laboratories Ltd.) or RPMI 1640 medium (Gibco Laboratories) supplemented with antibiotics, penicillin G (100 units/ml) (Sigma Chemicals), streptomycin sulfate (100μg/ml) (Sigma Chemicals) and 10% (v/v) fetal bovine serum (FBS) (Gibco, Ltd.). The formulation of α-MEM has been published by Stanners *et al.* (1971). Cells were incubated at 37°C in a humidity-controlled incubator containing a 5% CO_2 atmosphere (Wright, 1973).

1.2 Routine Cell Culture Procedures

Phosphate Buffered Saline:	137 mM sodium chloride
	2.68 mM potassium chloride
	1.62 mM potassium phosphate monobasic
	8.10 mM sodium phosphate dibasic
	pH 7.3

Trypsin treatment of cultured cells: To enzymatically detach cells from the surface of tissue culture plates, the medium was first removed by aspiration and the plates were washed once with 5ml of phosphate buffered saline (PBS). A 2 ml aliquot of a 0.3% trypsin (Bacto trypsin, Difco) in sterile PBS solution was added to the

culture plates. The trypsin treatment was carried out at room temperature and usually required 1-2 minutes. When the cells appeared detached, 2 ml of α-MEM containing 10% FBS was added to the plates to inhibit the trypsin activity. The cells were transferred into a sterile centrifuge tube and recovered by centrifugation at 500 X g for 5 minutes, and then resuspended in an appropriate medium.

Subculture: Cell cultures that approached confluence were subcultured. The cells were detached from the surface with the aid of trypsin solution and washed in α-MEM containing 10% FBS. The density of the cell suspensions was determined by the use of a hemacytometer (Hausser Scientific). An aliquot of 1 X 10^5 cells was transferred to a fresh 100mm tissue culture plate containing 10ml of α-MEM with 10% FBS.

Cold storage and resuscitation of frozen cells: For long term cold storage, a population of cells was resuspended in αMEM containing 10% FBS and 10% dimethyl sulfoxide (DMSO) (Fisher Scientific) at a density of about 5 X 10^6 cells per ml. Cells were frozen in 1 ml aliquots in a cryotube (Nunc) at -70°C. For reculturing of frozen cells, the contents of the cryotube were thawed quickly in a 37°C waterbath. The cells were washed once with α-MEM containing 10% FBS and then resuspended in the same medium for incubation in a culture plate.

1.3 Cell lines

Wild type mouse L cells: The mouse L cells used in this study were originally isolated by Earle (1943). This immortal mouse fibroblast cell line has been studied

extensively and used successfully to isolate a variety of mutant phenotypes (e.g. Dubbs & Kit, 1964; Thompson **et al.**, 1970,1971; Kuzik & Wright et al., 1980, 1983; and McClarty ***et al.***, 1986a, 1987a; Choy *et al.*, 1988).

A series of hydroxyurea resistant mouse L cell lines with gradually increasing drug resistance properties were isolated from the wild type population by culturing the wild type mouse L cells (L60) in the presence of increasing concentrations of drug starting from 0.35 to 30.0 mM (McClarty et al., 1986a; Choy *et al.*, 1988). A moderate hydroxyurea-resistant cell, which is resistant to 5.0 mM hydroxyurea has been called LHF in published studies (McClarty et el., 1986a,b; 1987a). A subclone of LHF cell, SC2 was used in this study and has been characterized in detail (McClarty *et al.*, 1986a, 1986b, 1987a, 1988). Two highly hydroxyurea resistant cells, HR-15 and the HR-30 are resistant to 15.0 mM and 30.0 mM of hydroxyurea, respectively. These two mutant cell lines have also been well studied (Hurta & Wright, 1990a,b). Unless otherwise stated, $SC2^-$/HR-30^- and $SC2^+$/HR-30^+ refer to drug resistant cell lines that were cultured in the absence or presence of 5mM hydroxyurea, respectively. The growth medium (with or without hydroxyurea) was routinely replaced with fresh medium every 24 to 48hr during experimentation. $SC2^-$ cells have a doubling time of approximately 25 hr, and $SC2^+$ cells doubled about every 32 hr. $SC2^-$ and HR-30^- cells were routinely cultured in the absence of hydroxyurea for at least two weeks prior to experimentation.

Human carcinoma cell lines : A2780S and A2780DDP are human ovarian carcinoma cell lines sensitive and resistant to cisplatin, respectively (Scanlon &

Kashani-Sabet, 1988; Lu *et al.*, 1988; and Scanlon *et al.*, 1989b). HCT8S and HCT8DDP are human colon carcinoma cell lines sensitive and resistant to cisplatin respectively (Chen & Scanlon, 1988 and Scanlon *et al.,* 1989a, 1989b). The above cell lines were kindly provided by Dr. K.J. Scanlon, Department of Medical Oncology, City of Hope National Medical Center, Duate, California. All cell lines were grown in RPMI 1640 medium supplemented with penicillin G, streptomycin sulfate and 10% FBS. A2780DDP cells were made resistant to cisplatin by weekly 1hr exposures to 50 μM cisplatin for 6 months (Lu *et al.*, 1988). A2780S and A2780DDP have a generation time of about 19 hr, respectively, when growing in RPMI 1640 medium with 10% fetal calf serum containing 10^{-8} M folinic acid. The A2780DDP cell lines exhibited 3-5 fold resistance to cisplatin (Lu *et al.*, 1988; Scanlon & Kashani-Sabet, 1988). The HCT8DDP cell line exhibited a 4.3 fold resistance to cisplatin (Chen & Scanlon, 1988).

2.0 Determination of protein Concentration

The concentration of protein in cell free preparations was measured using a Bio-Rad protein assay kit (Bio Rad Laboratories, Technical bulletin 1051). Purified bovine serum albumin (sigma Chemical Co.) was used to generate a protein standard curve covering the range of zero to 80 μg of protein.

3.0 Preparation and Assay of Ribonucleotide Reductase Activity

CDP assay mixture:
50 mM Tris/HCl pH 7.6
2 mM ATP
6 mM Dithithreitol (DTT)
8 mM $MgCl_2$
0.4 mM [^{14}C]-CDP (10,000 dpm/mole)

Ribonucleotide reductase activity in mouse L cells and human carcinoma cells was measured in cell-free preparation by the method described by Steeper and Stuart (1970), and subsequently modified by Cory *et al* (1973) and Lewis et al., (1978c). Exponentially growing cells were harvested and washed twice with PBS, pH 7.2. The cells were resuspended at approximately 8 X 10^6 cells per 200 μl of 1 mM DTT (Boehringer Mannheim) in 50mM Tris-HCl pH 7.6. The cell suspension was disrupted with 3 second pulses of sonication 3 times at 30% power, or by freezing and thawing for 2-3 times, and then centrifuged at about 11,000 rpm (Beckman Model J2-21 Centrifuge), at 4°C for 20 minutes to remove cellular debris. The supernatant (cell extract) was frozen at -70°C immediately. An aliquot (100-150 μg protein) of the cell extract was added to 25 μl of CDP reductase assay mixture. The final volume of the reaction was made up to 150 μl with assay buffer containing 1mM DTT, 1mM PMSF, 50mM Tris-HCl pH 7.6. The final concentrations of the essential reagents contained in the reaction were: 4mM magnesium acetate, 6 mM DTT, 1mM ATP (Sigma Chemical Co.,) and 50 μM [^{14}C]-CDP (486 mCi/m mol) (Moravek Biochemical, Inc. California).

After incubating at 37°C for 20 minutes, the reaction was stopped by boiling

for 4 minutes, followed by the addition of 50 μl (1mg) of *Crotalus atox* Venom (Sigma) with 10mM $MgCl_2$ in 50mM Tris-HCl pH 7.6. The nucleotides were converted to nucleosides by treatment for 1 hour at 37°C with *Crotalus atox* Venom. The reaction was stopped again by boiling for about 4 minutes. 0.5 ml of deionized water was then added to each reaction tube and the heat precipitable material was removed by centrifugation. The newly formed deoxycytidine was separated and measured by the method of Steeper and Steuart (1970). The supernatant was passed through a Dowex-1-borate column 5 X 80 mm. The deoxycytidine was eluted with 4 ml of deionized water and the eluate was mixed with 10 ml of Scintiverse II (Fisher Scientific) and counted with a liquid scintillation counter (Beckman, LS7800). Specific enzyme activity was expressed as nmoles of CDP reduced/hr/mg protein.

Enzyme inhibition assay: Cell extracts were prepared as above, CDDP (MW 300.06, 99.99% purity, Aldrich Chemical Co.) was freshly prepared before use by dissolving 1.5mg of CDDP in 25μl DMSO. CDDP was then diluted into various concentrations ranging from (0-1.0mM) using assay buffer. Pre-determined concentrations of diluted CDDP were incubated with 100-150 μg protein at room temperature for 10 min. CDP assay mixture was added to each of the reaction vials and the assay was continued as described above. Percent of enzyme inhibition was expressed as the % of specific enzyme activity in the presence of drug relative to control (no drug present).

4.0 Incubation of Ribonucleotide Reductase with CDDP Under

Anaerobically Enriched Conditions

To determine ribonucleotide reductase activity under highly reduced anaerobically enriched conditions, cell extract was prepared from $SC2^+$ cells and flushed with N_2 gas for about 5 min on ice. Various concentrations of CDDP were added to microfuge tubes containing cell extracts (about 150 μg protein). Enzyme was inhibited with CDDP under oxygen-free conditions, achieved by sparging each microfuge tube with N_2 gas in a N_2 saturated container. The incubation of cell extracts with CDDP was also carried out at room temperature and for 10 min. After inhibition, enzyme activity was determined by the CDP reductase activity assay as described above.

5.0 Preparation of Dowex-1-borate Resin

The ion exchange resin (AG1-X8) was purchased from Bio-Rad Laboratories Ltd. as Dowex-1-Chloride, 200-400 mesh and the chloride ions were replaced with borate ions. About 450 g of resin was resuspended in 4 liters of saturated sodium borate solution and stirred overnight at room temperature. The resin was collected by filtration and then resuspended in another 4 litres of saturated sodium borate solution. After overnight stirring , the resin was again collected by filtration and washed with 16 litres of deionized water. Finally, the Dowex-1-borate resin was suspended in about 500 ml of water to make a thick slurry and stored at 4°C.

6.0 Colony Forming Ability

After cell number was determined by a hematocytometer, a pre-determined number of cells ranging from 100 to 10,000 cells were added to 100mm dishes containing 10ml of growth medium. Dishes receiving 100 or 200 cells were used as controls or for relatively low drug concentrations. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (Wright, 1973). Next morning, selected drug (Cisplatin or Hydroxyurea [Sigma Chemical Co.]) was prepared freshly by dissolving the drug in the culture medium, sterilized through a 20 μm Nalgene filter unit, and added to the cultures at the desired concentrations. All assays were done in duplicate. After an incubation period of about 7 to 12 days at 37°C, the dishes were washed once with PBS and stained with a filtered 50% solution of ethanol saturated with methylene blue (Sigma Chemical Co.) at room temperature for about 15 minutes. Colonies containing more than 50 cells were counted. Plating efficiency was defined as the ratio of colonies relative to the number of cells plated.

The effect of various drugs on the growth of cells can be studied by determining the relative plating efficiency (RPE). The RPE is defined as the plating efficiency in the presence of drug divided by the plating efficiency in the absence of drug. The RPE was determined by adding a pre-determined number of cells in culture plates containing medium with increasing concentrations of drug, and in the absence of drug as a control (Hards & Wright, 1981).

7.0 Determination of Growth Rate

Exponentially growing cisplatin sensitive and resistant cells were removed from culture plates with buffered trypsin solution. Cells were washed with PBS and counted with the aid of a hematocytometer. A set of 60mm culture plates containing 5 ml of RPMI 1640 medium with 10% FBS was supplemented with 5 x 10^4 cells. After three hours incubation at 37°C, cells from two of the plates were independently harvested with trypsin solution and the total number of cells on each plate was determined. The number of cells determined was considered the number of cells at time zero. This ensures that the cells are in log phase of growth and minimizes the distortion caused by a lag period. At approximately every 12 hr interval, another two culture plates were harvested and the total number of cells on each plate were counted. The proliferation rates of the cells growing in the growth medium containing appropriate concentrations of hydroxyurea or no hydroxyurea were determined.

8.0 Affinity chromatography on blue dextran-sepharose

M1 and M2 proteins of ribonucleotide reductase were separated by affinity chromatography on blue dextran-Sepharose (Eriksson *et al.*, 1981a). The blue dextran-Sepharose column was synthesized (Ryan and Vestling, 1974) and kindly provided by Dr. J. Cory, School of Medicine, Department of Biochemistry, East Carolina University. The column (1 x 6 cm) was equilibrated by running at least 10x

column volume of buffer A (50 mM Tris-Cl buffer at pH 7.5 and 2 mM DTT) through the column. The whole separation procedure was carried out in the cold room (4°C). Cell extract (2 ml at 27 mg protein/ml) was applied onto the equilibrated column. The column was then washed with buffer A until no protein was detected when assayed with a Bio Rad Protein assay kit. The fraction was collected on the ice and designated as M2 fraction. Subsequently, the bound proteins were eluted with 10 ml of buffer B (1 M KCl, 50 mM Tris-HCl, pH 7.5, and 1 mM DTT), and the eluate was designated as M1 fraction. Both M1 and M2 fractions were concentrated to around 3 mg/ml by centrifugal ultrafiltration using centricon-10 (10,000 MW cutoff) microconcentrator (Amicon, Operating Instructions). Both fractions were then frozen away immediately at -70°C for further studies. The column was regenerated by washing with 20x column volume of 3M NaCl/50mM Tris-HCl as well as buffer A. The column was kept in buffer A with 0.1% sodium azide and in 4°C.

9.0 Western Blot Analysis

SDS-loading buffer:	3% (w/v) sodium dodecyl sulfate (SDS) 10% (v/v) glycerol 5% (v/v) ß-mercaptoethanol 0.05% (w/v) bromophenol blue 625 mM Tris-HCl pH 6.8
Tris-buffered saline (TBS):	50 mM Tris-HCl, pH 7.5 150 mM NaCl
TBS-Tween:	TBS 0.5% Tween 20 (Sigma Chemical Co.)

Carbonate-$MgCl_2$ buffer:	80 mM sodium bicarbonate 1 mM $MgCl_2$

Following cell extract preparation, a predetermined quantity of total cellular protein was heated at 100°C for 2 min in SDS loading buffer and then analyzed on a 10% linear SDS-polyacrylamide gel as previously described (Engstrom *et al.*, 1979; McClarty *et al.*, 1987a). Proteins were then transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). This transfer was carried out at 50 volts and room temperature for 1 hour. After transfer, membranes were blocked in 50mM Tris-HCl (pH 7.6) in saline (TBS) containing 0.5% Tween 20 (TBS-Tween) plus 1% BSA for 1 hour. The membranes were then incubated with either AD203 anti-M1 mouse monoclonal antibody (Engstrom *et al*,. 1984) or JB4 anti-M2 rat monoclonal antibody (McClarty *et al.*, 1987a) for 3 hours at room temperature or overnight at 4°C on the nutator (Clay Adams). The membranes were then washed 3 times for 30 min each in TBS-Tween followed by incubation with the appropriate second antibody for 1 hour at room temperature. Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was used for M1 detection, and rabbit anti-rat IgG conjugated with alkaline phosphatase (Sigma) was used for detecting protein M2. Following incubation with second antibody, the blots were washed three times for 30 min each in TBS-Tween. Finally, the bound antibodies were visualized by the development of the alkaline phosphatase reaction, as described by Blake *et al.* (1984) in carbonate $MgCl_2$ buffer.

10. 0 Northern Blot Analysis

10.1 Isolation of Total RNA by Guanidine Isothiocyanate (GITC)

Guanidinium lysis buffer:	4M GITC 25mM sodium citrate (pH 7.5) 0.5% N-laurylsarosine 0.1% Anti-foam A (Sigma Chemical Co.) 0.007% ß-mercaptoethanol (added immediately before use)
CsCl buffer:	5.7 M CsCl 0.1 M EDTA (pH 7.0)
RNA water:	0.01% diethyl pyrocarbonate (DEPC) in deionized water and autoclaved 15 min, 120°C

Total cellular RNA was extracted from logarithmically growing cells using the guanidinium isothiocyanate/cesium chloride method (Chirqwin et al., 1979). Cells were harvested from 6 to 8, 150mm culture plates, washed once with PBS and centrifuged. The pellet of approximately 10^8 cells was then resuspended in 2 ml of guanidinium lysis buffer. The suspension was then drawn through an 18 gauge syringe approximately 20 times to hydrodynamically shear the DNA. The resultant foaming of the lysis buffer was cleared or reduced by a 15 min. centrifugation in a clinical centrifuge. The cell lysate was diluted up to 9 ml with the guanidinium lysis buffer and layered on top of a 3.0 ml cushion of CsCl buffer in a Beckman SW41 centrifuge tube. The suspension was then centrifuged at 20°C in a SW41 Ti Beckman rotor at 25,000 rpm (180,000 x g) for 18 hours. The top guanidinium isothiocyanate layer and the CsCl cushion including the band of DNA were removed with a pasteur pipette,

and the tube was cut slightly above the pellet. The sides of the centrifuge tube and the pellet were washed once with guanidinium lysis buffer, and then twice with 70% ethanol plus 0.1% SDS. The RNA pellet was resuspended in 500 μl sterile RNA water, transferred to a microfuge tube and precipitated overnight at -20°C by the addition of 1/10 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol. The sample was then centrifuged at 14,000 x g and 4°C for 10-15 minutes in a benchtop microcentrifuge. The pellet containing total cellular RNA was then resuspended in 100 to 150 μl of sterile RNA water and stored at -70°C.

The concentration of RNA was determined by usually diluting 5 μl of RNA in 995 μl deionized water, and measuring the absorbance at both 260 and 280 nm. Only those preparations having $OD_{260\lambda}/OD_{280\lambda}$ ratios of 1.8 to 2.1 were used. Using the formula: 1 $OD_{260\lambda}$ = 40 μg/ml RNA, when the 260λ/280λ ratio is 2.0 (Maniatis *et al.*, 1982), allows for calculation of the RNA concentration.

10.2 Northern Blotting

5x MOPS buffer:	0.1 M morpholinopropanesulfonic acid (pH 7.0) 40 mM sodium acetate 5 mM EDTA
6x RNA Gel Loading buffer:	0.25% bromphenol blue 0.25% xylene cyanol 1 mM EDTA (pH 8.0) 50% glycerol
1 X SSC	0.15 M NaCl 0.015 M sodium citrate

pH 7.0

The RNA was prepared for Northern blot analysis as follows: to 4.5 μl RNA sample containing 20 μg of cellular RNA was added 2.0 μl MOPS buffer, 3.5 μl formaldehyde and 10.0 μl formamide. The sample was then chilled on ice and 2.0 μl of 6 x RNA gel loading buffer was added. The sample was then loaded onto a 1% formaldehyde-agarose gel containing 18% formaldehyde and 1 x MOPS (Lehrach *et al.*, 1977; Miller, 1987), and electrophoresed overnight in 1 x MOPS buffer at 30 volts. An RNA sample containing ribosomal RNA was loaded into a separate lane as a relative mobility reference (Rf) marker. The marker lane was cut from the gel and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30-45 minutes and then destained with distilled deionized water for 30-45 minutes. The marker RNA bands (28s and 18s ribosomal RNA) were visualized, and the Rf measured under ultraviolet illumination. The remainder of the gel was blotted onto a nylon membrane (Zeta-probe, Bio-Rad, Laboratories Inc.) using 10 x SSC as a transfer buffer.

10.3 Hybridization/Autoradiography

After transfer, the membrane was removed from the gel, rinsed in 2-6 x SSC, air dried, and then baked at 80°C for 2 hours in a vacuum gel drier to fix the DNA to the membrane. The blots were prehybridized for 3 hours to overnight at 42°C in hybridization buffer supplemented with 10% (v/v) dextran sulfate (Sigma Chemical

Co.) and 600 μg/ml boiled single stranded salmon sperm DNA (Sigma Chemical Co.). Hybridizations were performed in the same solution for 16 hours with 10^6 counts per minute (cpm)/ml of ^{32}P-labeled *Nco*I-generated fragment containing the cDNA of clone 65 (M1 protein) or the *Pst*I fragment of clone 10 (M2 protein) (Thelander and Berg, 1986). cDNA probes were labeled by the hexadeoxyribonucleotide method of Feinberg and Vogelstein (1983) using [α-^{32}P]dCTP (specific activity 3000 Ci mmole^{-1}, Amersham) to a specific activity of 5 x 10^8 to 1 x 10^9 cpm/μg.

The blots were washed twice in 2 x SSC and 0.1% SDS at room temperature for 20 minutes each, and then washed twice with 0.1 to 0.5 x SSC and 0.1% SDS for 30 minutes each. The membranes were then autoradiographed at -70°C using Kodak X-Omat AR film and Cronex lightning Plus intensifying screens (DuPont).

Hybridized probe was stripped from the blots by washing twice for 30 min each in 50% formamide in 0.1 x SSC at 75°C and then rinsed in 0.1 x SSC. Loading of RNA was determined by reprobing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Edwards *et al.*, 1985; Choy *et al.*, 1989).

11.0 Statistical Analysis

Results were analyzed by covariates analysis of variants or by two way anova analysis of variants.

RESULTS

1.0 Effects of CDDP on Mammalian Ribonucleotide Reductase Activity

It has been published that ribonucleotide reductase purified from *E.coli* is strongly inhibited by CDDP (Smith & Douglas, 1989). Since ribonucleotide reductase plays a very important role in catalysing a rate-limiting step for DNA synthesis, it is an acknowledged anticancer target enzyme (Weber, 1983). Similar to *E.coli* ribonucleotide reductase, mammalian ribonucleotide reductase also has a pair of redox-active dithiols at the active site of the M1 subunit, and therefore it should be a favourable target for CDDP.

The experiment described below was carried out with the purpose of finding the effect of CDDP on mammalian ribonucleotide reductase activity. In previous studies (McClarty et al., 1987a, 1988; Hurta and Wright, 1990a), hydroxyurea-resistant mouse L cell lines, SC2 and HR-30 exhibited an elevation in ribonucleotide reductase activity due to altered regulatory mechanisms (see Introduction). Upon drug challenge, these cell lines showed a further elevation in enzyme activities (McClarty *et al.*, 1988; Hurta & Wright, 1990b). Wild type mouse L cells (L60), moderate hydroxyurea-resistant mouse L cells ($SC2^{+/-}$) and highly hydroxyurea-resistant mouse L cells ($HR\text{-}30^{+/-}$) were used in the study. The minus sign referred to cells growing in serum supplemented αMEM growth medium containing no hydroxyurea; while the plus sign referred to cells cultured in growth medium containing 5mM hydroxyurea for 2-3 weeks (McClarty et al., 1988). Cell extracts of these lines were prepared as

described in Materials and Methods. Ribonucleotide reductase activities for L60, $SC2^+$, $SC2^-$, $HR\text{-}30^+$ and $HR\text{-}30^-$ cells in the presence of varying concentrations of CDDP, were determined by the CDP reduction inhibition assay as described in Materials and Methods.

The result is shown in Figure 8. As indicated in this figure, CDDP inhibited CDP reductases from both L60 and $SC2^-$ cells with an approximately the same inhibitory effect. The 50% inhibition of CDP reductase prepared from L60 and $SC2^-$ cells occurred at about 0.29 mM CDDP.

Since hydroxyurea-resistant cell lines, $SC2^-$, $SC2^+$, $HR\text{-}30^-$ and $HR\text{-}30^+$ exhibited different M1 and M2 protein levels (Hurta & Wright, 1990a; McClarty *et al.*, 1986a,1988), it was interesting to explore the effects of CDDP on the enzyme activities from these four mutant cell lines. As shown in Figure 9, CDP reductases from the four hydroxyurea-resistant cell lines were inhibited by CDDP as expected, from the results shown in Figure 8. The 50% inhibition for $SC2^-$, $SC2^+$, $HR\text{-}30^-$ and $HR\text{-}30^+$ occurred at concentrations of 0.29 mM, 0.50 mM, 0.56 mM and 0.73 mM, respectively. By comparing their CDDP concentrations at 50% inhibition, it was shown that ribonucleotide reductases prepared from four different cells exhibited slightly different inhibitory responses. There was not a great deal of difference in enzyme sensitivity to CDDP inhibition when the results obtained with ribonucleotide reductase from $SC2^+$ cells was compared to results obtained with enzyme from $HR\text{-}30^-$ cells, where CDDP concentrations giving 50% inhibition were found to be 0.5 mM and 0.56 mM, respectively. However, about a 2.5 fold higher drug concentration was

Figure 8. Inhibitory effects of various concentrations of cisplatin on ribonucleotide reductase activity from wild type L60 cells and moderate hydroxyurea-resistant SC2^{-} cells grown in absence of hydroxyurea expressed as percent of activity in the absence of cisplatin.

CDP reductase activity was measured as described in Materials and Methods.

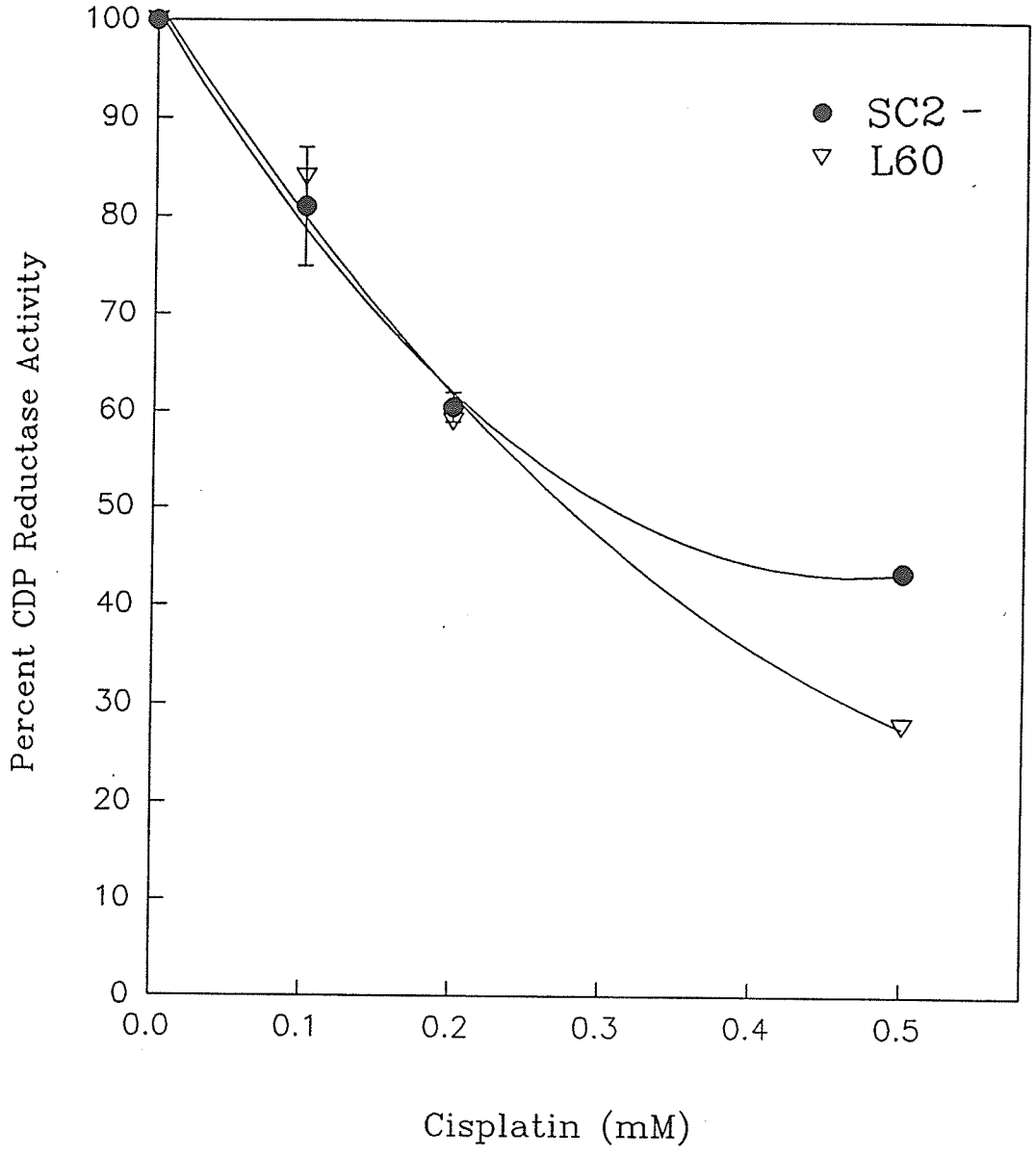
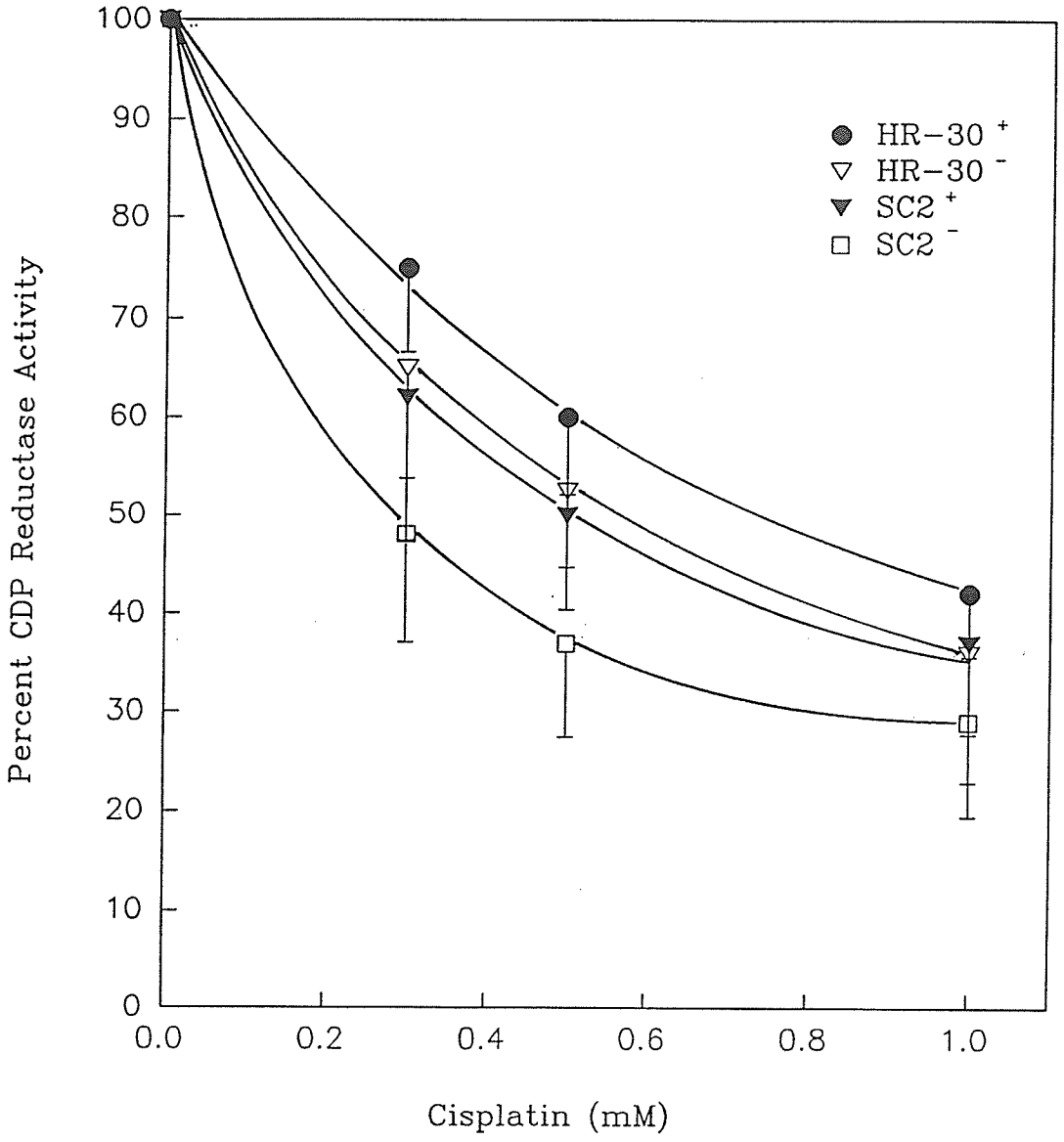

Percent CDP Reductase Activity
100
90
80
70
60
50
40
30
20
10
0
0.0
0.1
0.2
0.3
0.4
0.5
Cisplatin (mM)
SC2 -
L60

Figure 9. Inhibitory effects of various concentrations of cisplatin on ribonucleotide reductase activity from moderate hydroxyurea-resistant cells grown in the absence of hydroxyurea, $SC2^-$ (□) and grown in the presence of 5 mM hydroxyurea, $SC2^+$ (▽); and highly hydroxyurea-resistant cells grown in the absence of hydroxyurea, $HR\text{-}30^-$ (▽) and grown in the presence of 5 mM hydroxyurea, $HR\text{-}30^+$ (●), expressed as percent of activity in the absence of cisplatin.

CDP reductase activity was measured as described in Materials and Methods.

For each cell line, each data point was an average of at least 8 independent determinations.


Percent CDP Reductase Activity
100
90
80
70
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Cisplatin (mM)
HR-30 +
HR-30 -
SC2 +
SC2 -

required to inhibit CDP reductase activity from HR-30$^+$ cells by 50% than enzyme prepared from SC2$^-$ cells.

2.0 Inhibition of Ribonucleotide Reductase Under Aerobic and Anaerobically Enriched Conditions

As demonstrated in our study, like the ribonucleotide reductase from *E.coli* (Smith & Douglas, 1989), mammalian ribonucleotide reductase can also be inhibited by CDDP. It was believed that CDDP inhibited the ribonucleotide reductase from *E.coli* by interacting with SH groups at the active site. We therefore proposed that CDDP would interact with mammalian ribonucleotide reductase by a similar mechanism because of the structural similarities between the bacterial and mammalian enzyme (Thelander & Reichard, 1979; Whitfield & Youdale, 1989). Also, it was observed that pretreatment of the *E.coli* enzyme with nitrogen gas to obtain a fully reduced condition, resulted in a further increase in ribonucleotide reductase sensitivity to CDDP inhibition (Smith & Douglas, 1989). Preparation of mammalian ribonucleotide reductase and assays of its activity are routinely carried out under reducing conditions (in the presence of DTT). However, since the E.coli enzyme exhibited a greater sensitivity to CDDP when pretreated with nitrogen gas, we also performed inhibition studies with mammalian enzyme exposed to nitrogen gas, to be certain that the enzyme was fully reduced, and to parallel the conditions used in the previous study (Smith & Douglas, 1989). Anaerobically enriched cell extract was

prepared and enzyme incubation in the presence of CDDP was carried out under N_2 conditions as described in Materials and Methods. After pre-incubation of the enzyme with CDDP, enzyme activity was measured by the CDP reductase assay as described in Materials and Methods.

The results were shown in Figure 10. Clearly, in the mammalian situation , pretreatment with nitrogen did not significantly alter the drug sensitivity of the enzyme, since 50% inhibition was still observed at approximately 0.3 mM CDDP.

3.0 Separation of Proteins M1 and M2 by Affinity Chromatography on Blue Dextran-sepharose

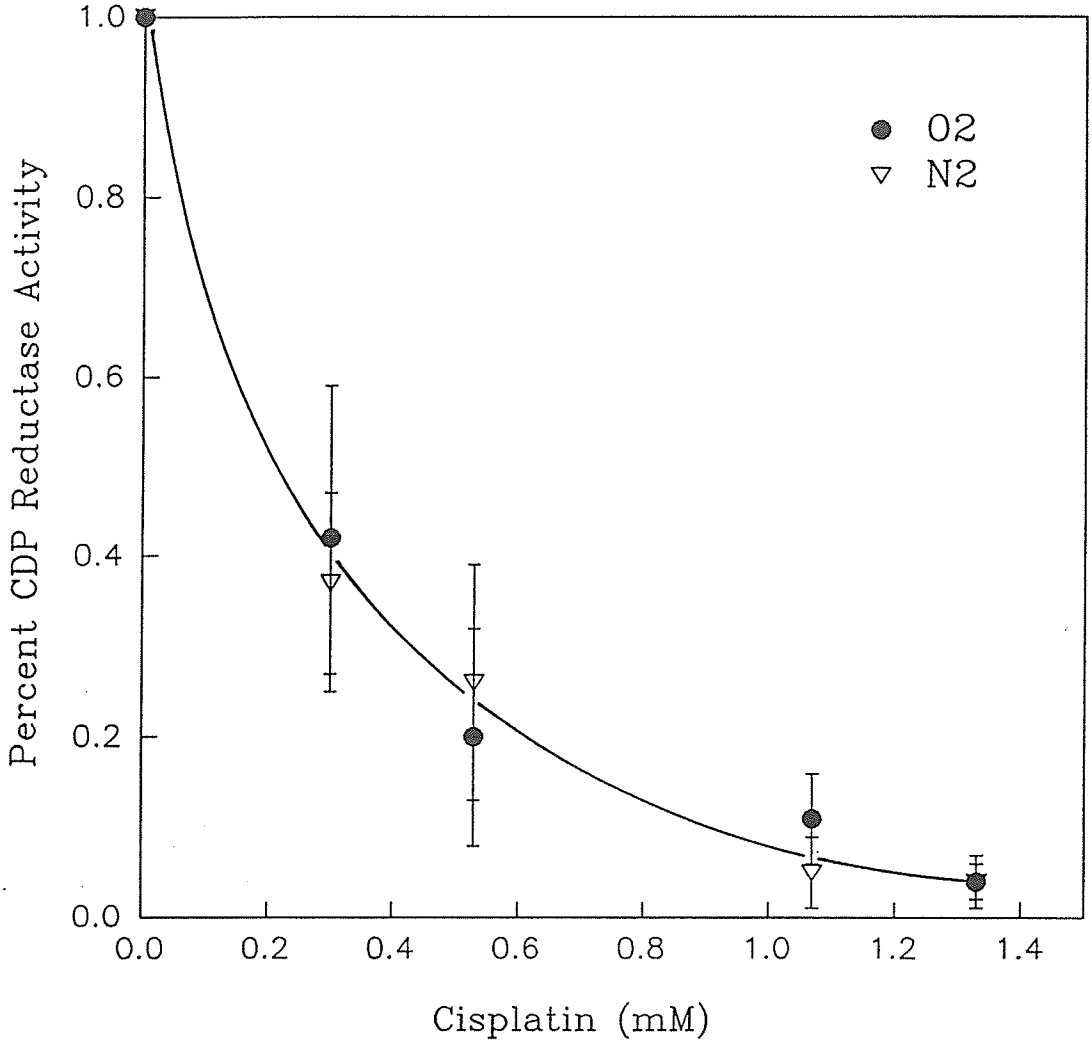
Cell extract prepared from either HR-30$^+$ or SC2$^+$ cells was passed through the blue dextran-sepharose column to separate M1 protein from M2 protein as described in Materials and Methods. The blue dextran on the column specifically binds to proteins possessing the dinucleotide fold (Thompson, *et al.*, 1975), such as the M1 protein of mammalian ribonucleotide reductase (Eriksson *et al.*, 1981a). By this affinity chromatography, the M1 and M2 proteins were separated into the Tris buffer fraction (M2 fraction) and the eluate fraction (M1 fraction), respectively as described in Materials and Methods.

Enzyme activity in each individual fraction was determined following separation. As shown in Figures 11A and B, separation of enzyme subunits was

Figure 10. Inhibition of ribonucleotide reductase with cisplatin under aerobic (●) and anaerobically enriched conditions (∇).

Cell extract used for enzyme assay was prepared from $SC2^+$ cells.

Each data point was the average of at least 2 independent determinations. No statistically significant differences in slope or intercept were observed when inhibition under the two conditions was analysed.


1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Percent CDP Reductase Activity
Cisplatin (mM)
O2
N2

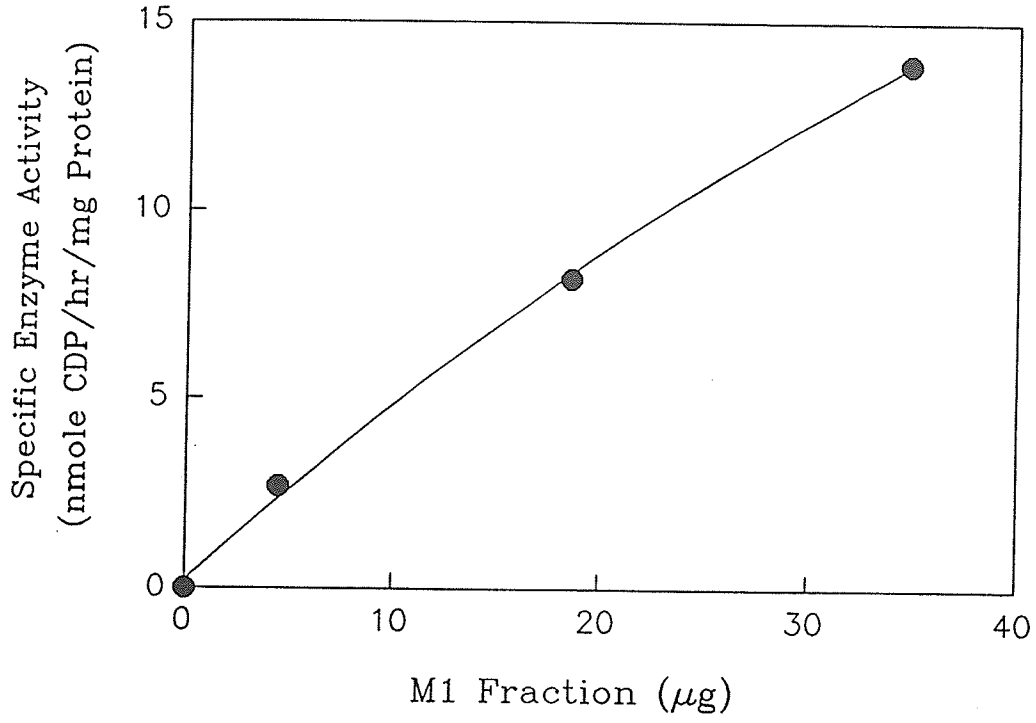
successfully achieved. Neither the M1 fraction nor the M2 fraction exhibited any ribonucleotide reductase activity alone (Figure 11A,B), but when combined, they gave full recovery of the enzyme activity. The figures demonstrated that ribonucleotide reductase activity was dependent upon the addition of either the M1 protein containing fraction to a fixed amount of M2 protein containing fraction (84μg) (Figure 11A), or the addition of the M2 protein containing fraction to a fixed amount of M1 protein containing fraction (34μg) (Figure 11B).

4.0 Effects of CDDP on M1 and M2 Proteins

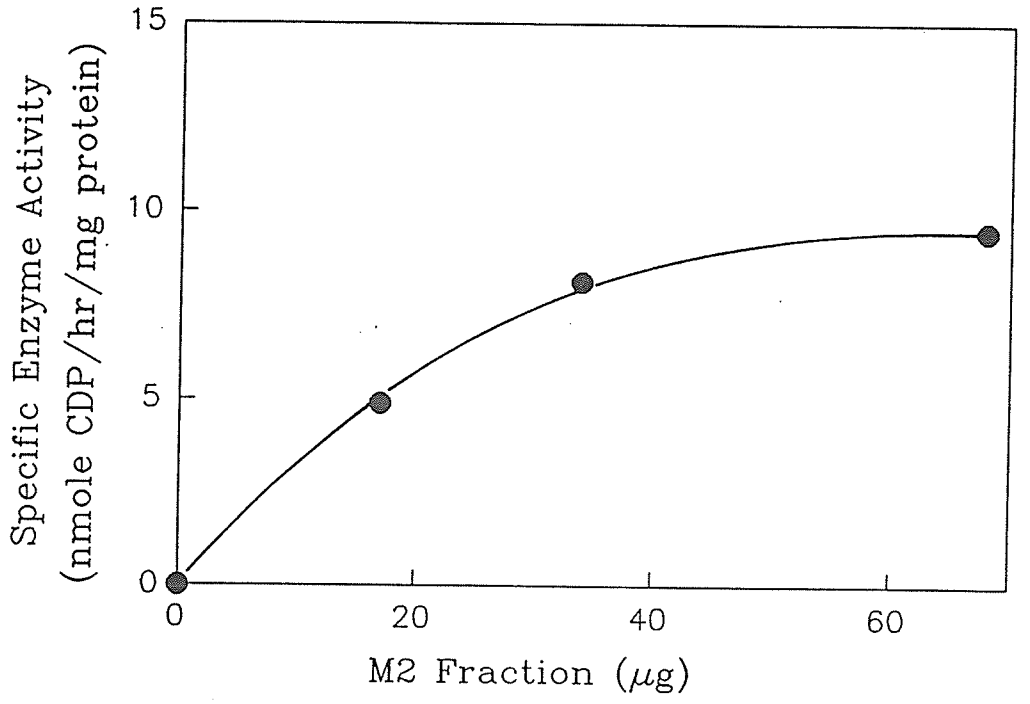
In order to dissect the mechanism by which CDDP inhibited the mammalian ribonucleotide reductase, the holoenzyme was separated into its two subunits, M1 and M2. The purpose of this experiment was to study the effects of CDDP on each component of ribonucleotide reductase alone and hence to reveal the target site of inhibitory action. CDDP was incubated separately with either the M1 or M2 subunit. The experiment was performed as follows: M1 or M2 fractions collected from the blue dextran-sepharose column, were incubated with various concentrations of CDDP ranging from 0 to 2 mM at room temperature for 10 min. Excess or unreacted free CDDP was then removed from the incubation mixture by running it through a G-25 sephadex column. By this gel filtration process, proteins with large molecular weights passed through the gel after a quick spin using a clinical centrifuge (300 x g), however

Figure 11. Reconstruction of ribonucleotide reductase activity by adding increasing amounts of M1 protein to a constant amount of M2 protein (11A); or by adding increasing amounts of M2 protein to a constant amount of M1 protein (11B).

Note, in figure 11A, enzyme preparation was obtained from HR-30$^+$ cells, while in figure 11B, the enzyme was prepared from SC2$^+$ cells.


Specific Enzyme Activity
(nmole CDP/hr/mg Protein)
15
10
5
0
0
10
20
30
40
M1 Fraction (μg)

(A)


Specific Enzyme Activity
(nmole CDP/hr/mg protein)
15
10
5
0
0
20
40
60
M2 Fraction (μg)

(B)

the unreacted CDDP was trapped inside the beads. The fact that CDDP was trapped in the gel after spinning, was confirmed by testing this procedure with Phenol Red (M.W. 354.39). In addition, the CDDP-ribonucleotide reductase chelate from *E.coli* has previously been shown to be stable to the filtration process (Smith & Douglas, 1989).

The repurified subunit was individually assayed for ribonucleotide reductase activity using untreated complementary subunit. For example, reisolated M1 (50 μg of protein) from the CDDP-M1 incubation mixture was assayed using untreated M2 protein (64 μg of protein). Similarly, reisolated M2 (64 μg of protein) from the CDDP-M2 incubation mixture was assayed using untreated M1 protein (48 μg of protein). In this way, it was found that inhibition occurred by reaction of cisplatin with either the M1 or M2 subunit. Figure 12A shows that ribonucleotide reductase was inhibited when M1 subunit alone was treated with CDDP, and 50% inhibition occurred at a concentration of 1.25 mM. Figure 12B shows that ribonucleotide reductase was also inhibited when M2 subunit alone was treated with CDDP and 50% inhibition occurred at a concentration of 0.82 mM. These results demonstrate that both M1 and M2 proteins could be the sites of action for CDDP.

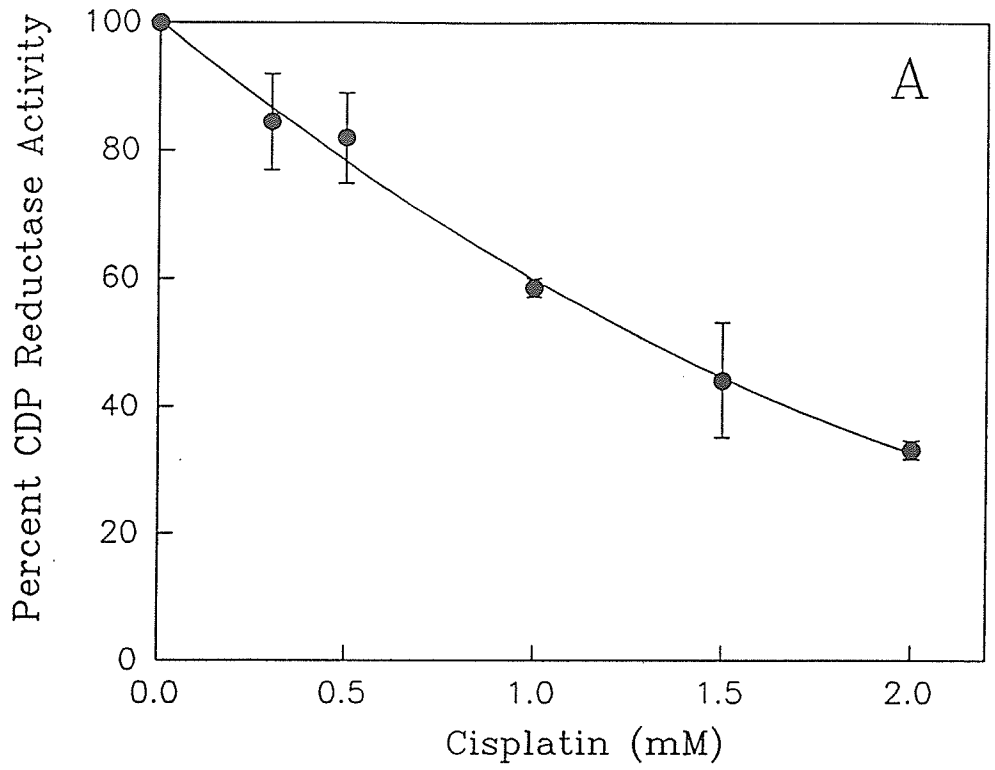
5.0 Colony-forming Ability in the Presence of CDDP

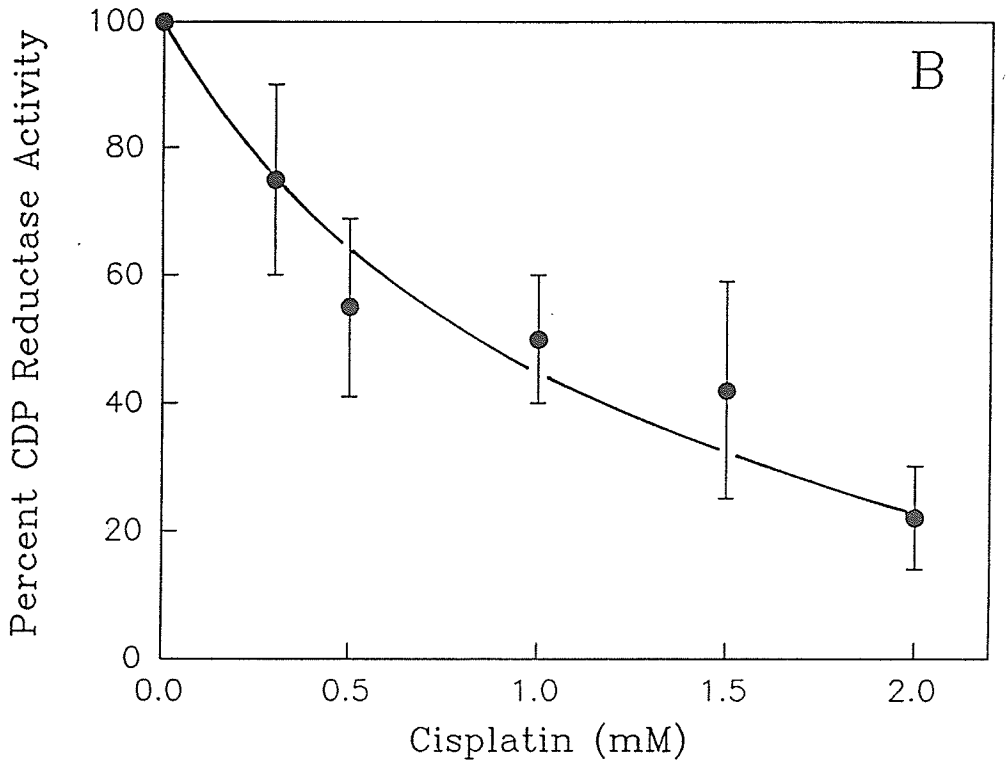
The cytotoxic effects of CDDP on wild type mouse L cells and hydroxyurea

Figure 12. Inhibition of ribonucleotide reductase by incubating various concentrations of cisplatin with M1 subunit (A) or M2 subunit (B).

M1 and M2 subunits were prepared by running the cell-free preparation through the blue dextran-sepharose affinity column. 60 μg of M1 fraction or 128 μg M2 fraction was incubated with the indicated concentrations of CDDP.

Each data point was an average of either 2 (A) or 3 (B) independent determinations.


A
Percent CDP Reductase Activity
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Cisplatin (mM)


B
Percent CDP Reductase Activity
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Cisplatin (mM)

resistant cells were compared by examining their abilities to form colonies when they were cultured in the presence of various concentrations of CDDP.

Figure 13 shows the RPE of wild type cells and the hydroxyurea resistant cell lines, $SC2^-$ and $SC2^+$ in the culture medium containing various concentrations of CDDP. The D_{10} values (the concentration of drug at which the RPE is ten percent of the control) for the wild type, $SC2^-$ and $SC2^+$ cells were 1.35 μM, 1.35 μM and 1.45 μM, respectively.

Figure 14 shows the RPE of wild type cells and the highly hydroxyurea resistant cells, HR-30^- and HR-30^+, in the same culture medium containing various concentrations of CDDP. The D_{10} values for the wild type cells, HR-30^- and HR-30^+ cells were 1.35 μM, 1.40 μM and 1.85μM, respectively. Futhermore, no statistically significant differences ($p > 0.05$ at all CDDP concentration tested) in the sensitivity to CDDP were observed when parental wild type cells (L60) were compared to the hydroxyurea resistant ribonucleotide reductase overproducing cell lines ($SC2^-$, $SC2^+$, HR-30^- and HR-30^+).

6.0 M1 and M2 Protein Levels in Wild Type Mouse L cells and Hydroxyurea-resistant Cells

It has been previously reported that hydroxyurea-resistant cell lines, SC2 and HR-30 exhibited a stable elevation in their ribonucleotide reductase activities

Figure 13. Relative plating efficiency of wild type L60 cell line (●) and moderate hydroxyurea-resistant cell lines, $SC2^-$ (▼) and $SC2^+$ (▽), in the absence and presence of various concentrations of cisplatin. The y-axis scale is logarithmic.

Each data point for the $SC2^-$ or $SC2^+$ cell lines was an average of at least 2 independent determinations, and each data point for the L60 cell line came from a single determination.

No statistically significant differences in slope or intercepts were observed when results obtained with these three cell lines were analysed.

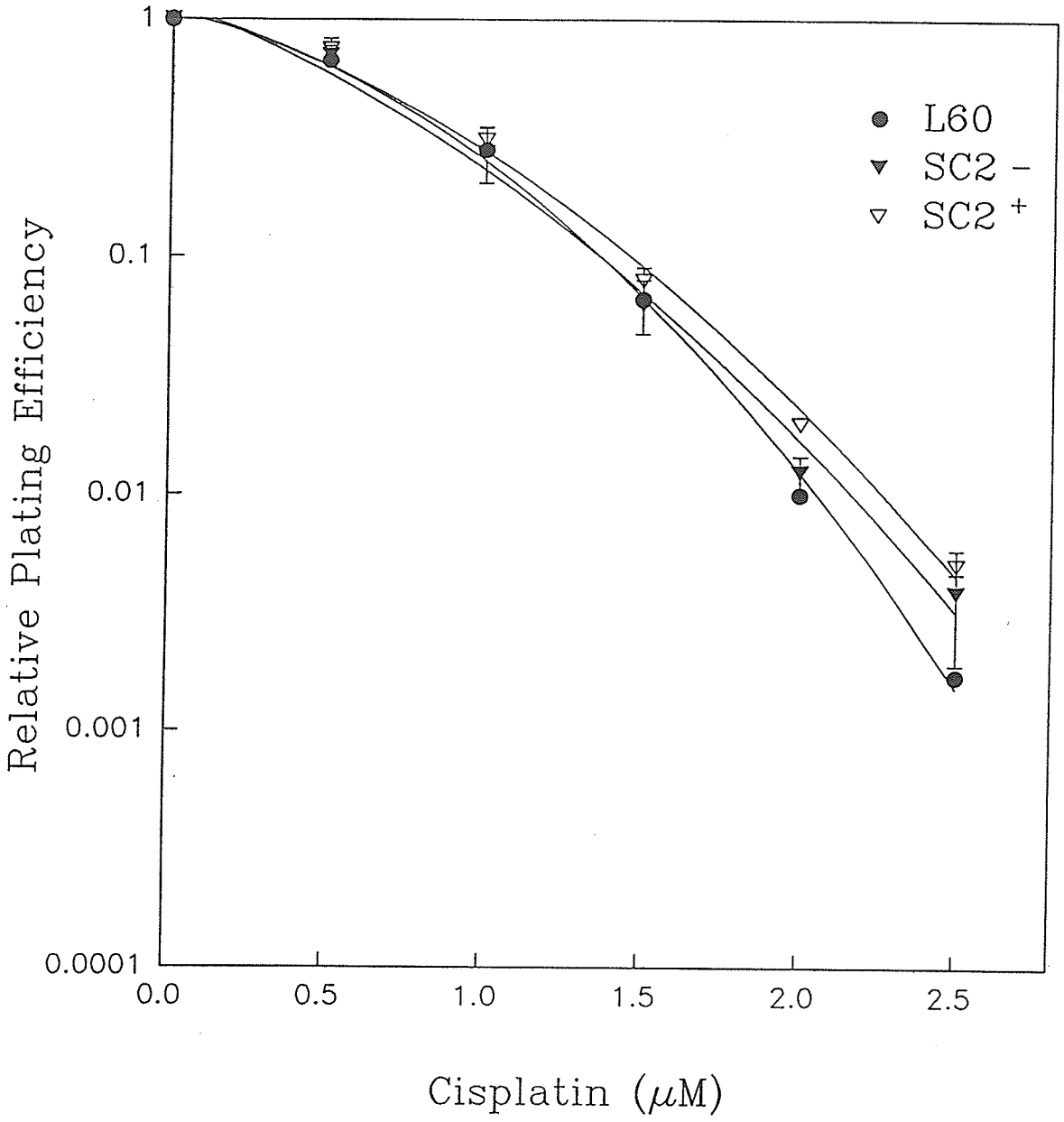
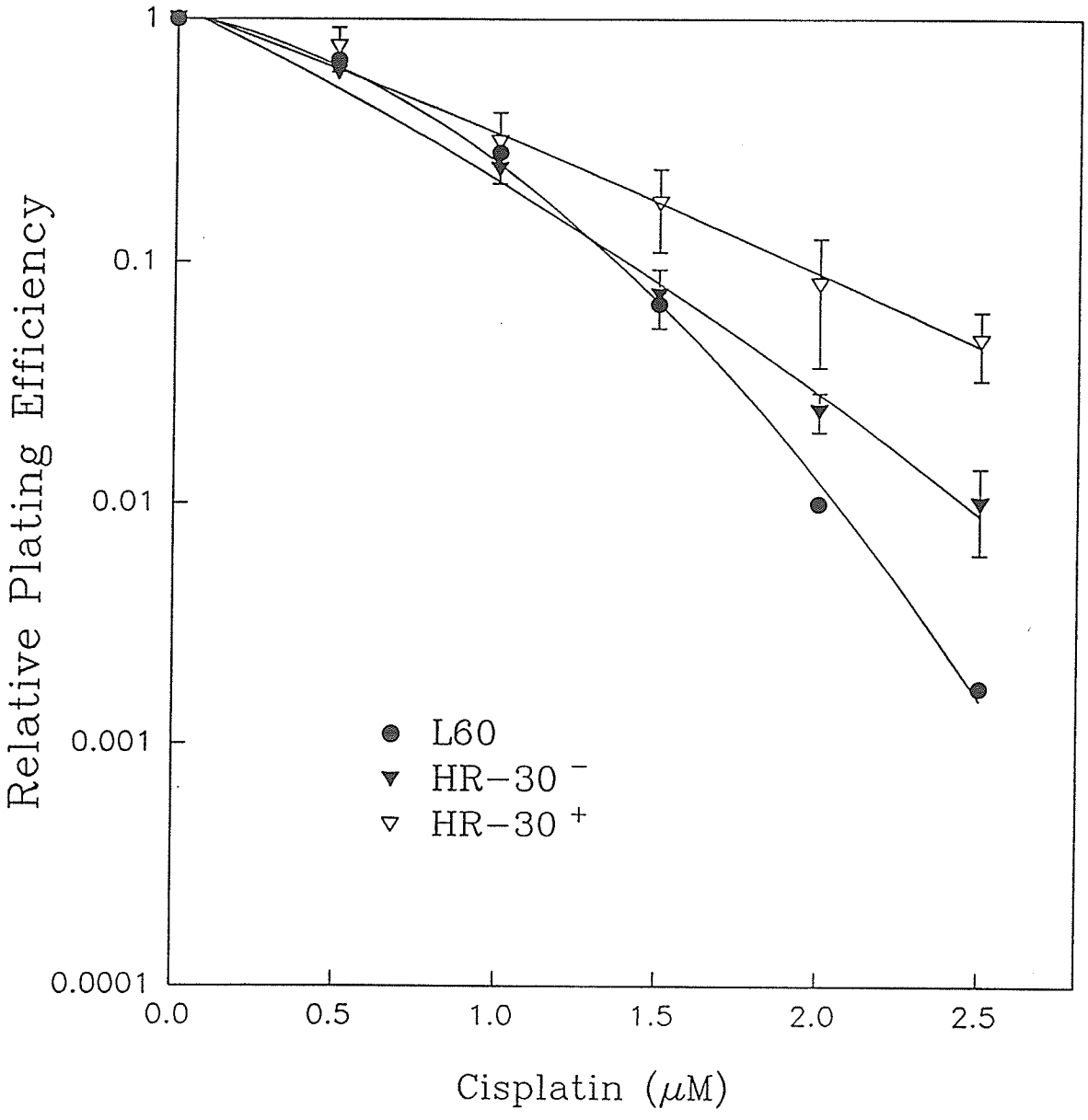

Relative Plating Efficiency
1
0.1
0.01
0.001
0.0001
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin (μM)
L60
SC2 −
SC2 +

Figure 14. Relative plating efficiency of wild type L60 cells (●) and highly hydroxyurea-resistant cells, HR-30$^-$ (▼) and HR-30$^+$ (▽), in the absence and presence of various concentrations of cisplatin. The y-axis scale is logarithmic.

Each data point for HR-30$^-$ or HR-30$^+$ cell line was an average of at least 2 independent determinations, and one determination was obtained with the L60 cell line.

No statistically significant differences in slope or intercepts were observed when results obtained with these three cell lines were analysed.


Relative Plating Efficiency
1
0.1
0.01
0.001
0.0001
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin (μM)
L60
HR−30 −
HR−30 +

(McClarty *et al.*, 1987a; Hurta & Wright, 1990a,b). Table 1 summarizes the genetic properties of the moderate ($SC2^-$) and highly ($HR\text{-}30^-$) hydroxyurea-resistant cell lines. It has also been demonstrated that the ribonucleotide reductase activity was further increased upon the addition of hydroxyurea to the culture medium (McClarty *et al.*, 1987a). For example, SC2 cells growing in the presence of 5 mM hydroxyurea showed a further 4-fold increase in enzyme activity, which was a result of a further increase in both protein M1 and M2 levels. However, neither the levels of M1 or M2 transcripts, nor the numbers of M1 or M2 gene copies were changed when the SC2 cells were grown in the presence of 5 mM of hydroxyurea.

Since, at this point the putative target for the action of CDDP on the reductase is M1 as well as M2 subunit, it was of interest to examine the cellular level of M1 and M2 protein levels in the wild type L60 cell line and the hydroxyurea-resistant cell lines.

As described in Materials and Methods, cell-free preparations obtained from wild type, $SC2^-$, $SC2^+$, $HR\text{-}30^-$ and $HR\text{-}30^+$ were subjected to SDS-PAGE and blotted onto nitrocellulose for Western blot analysis. Protein M1 and M2 were detected by using anti-M1 and anti-M2 monoclonal antibody, respectively. Protein M1 levels were compared in wild type, $SC2^-$, $SC2^+$, $HR\text{-}30^-$ and $HR\text{-}30^+$ (Figure 15). The results indicated that SC2 cells grown in the absence of hydroxyurea contained more M1 protein than did wild type cells. Also shown in the figure are the results of a comparison of protein M1 levels in SC2 and HR-30 cells grown in the presence and absence of hydroxyurea. The data clearly indicated that there was a dramatic increase

Table 1. Biochemical and Molecular Characteristics of Hydroxyurea-resistant Cell lines

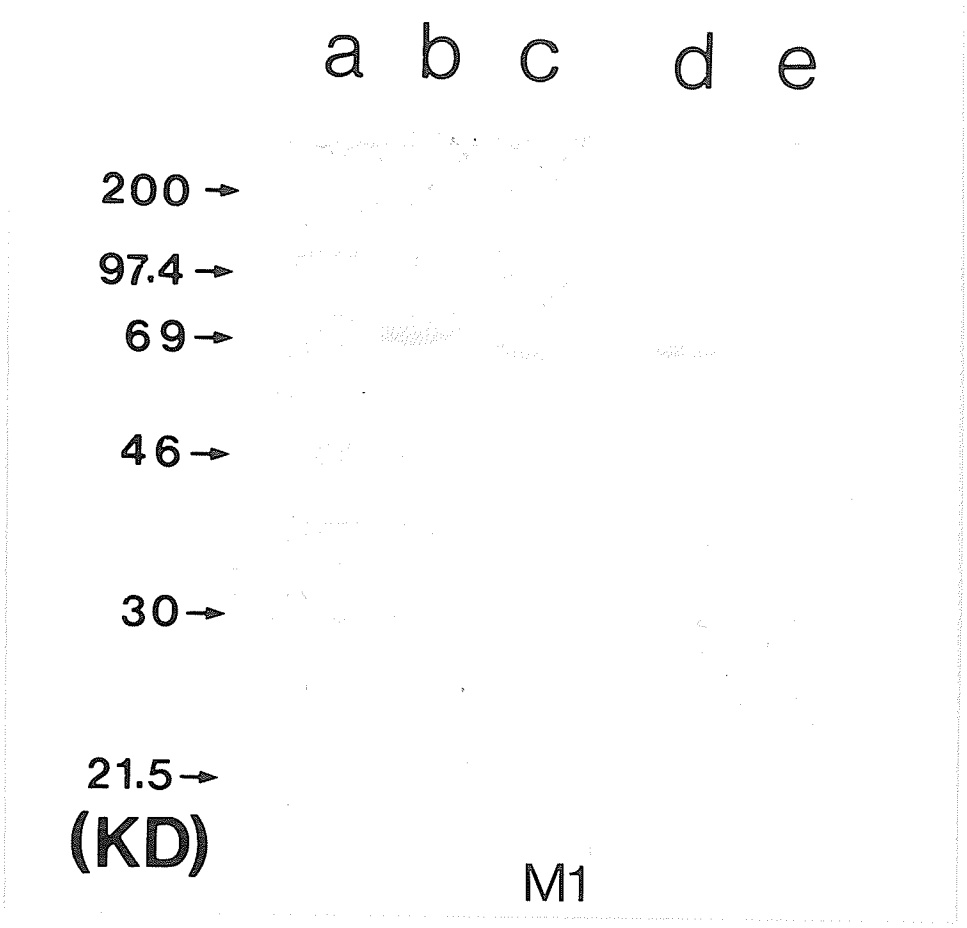
Cell Lines	Relative enzyme activity	Relative M1 protein levels[d]	Relative M2 protein levels[d]	Relative M1 DNA hybridization[e]	Relative M2 DNA hybridization[e]	Relative M1 RNA hybridization[f]	Relative M2 RNA hybridization[f]
SC2-[a]	5.0	2.0-3.0	50.0	1.0	6.0	2.0-3.0	35.0-40.0
HR-30-[b]	23.0	15.0-20.0	100.0	4.0	12.0-14.0	10.0	150.0

[a] Results obtained from McClarty *et al.*, 1987a.
[b] Results obtained from Hurta & Wright, 1990b.
[c] Enzyme activity was measured as nmole CDP reduced/hr/mg protein and expressed relative to the wild type result.
[d] Determined from densitometric scanning of data from Western blots, using M1 or M2 specific monoclonal antibodies and total protein from wild-type and hydroxyurea-resistant cells. Results are expressed relative to the wild-type situation.
[e] Determined from densitometric measurements of appropriate autoradiograms of the most prominent band hybridizing with M1 or M2 cDNA from drug-resistant cells, relative to measurements of the most prominent band hybridizing with DNA from wild-type cells.
[f] Determined from densitometric scanning of appropriate autoradiograms of RNA from drug-resistant and wild-type cells and expressed relative to the wild-type result.

Figure 15. Western blot analysis for total cellular M1 protein levels in wild type (a), HR-30$^+$ (b), HR-30$^-$ (c), SC2$^+$ (d), and SC2$^-$ (e) cell lines.

Protein preparation and the Western blot procedures were carried out as described under Materials and Methods.

Lane: (a) wild type, 120 μg of protein; (b) HR-30$^+$, 60 μg of protein; (c) HR-30$^-$, 60 μg of protein; (d) SC2$^+$, 60 μg of protein; and (e) SC2$^-$, 60 μg of protein.


a b c d e
200
97.4
69
46
30
21.5
(KD)
M1

in subunit M1 levels in $SC2^+$ cells compared to $SC2^-$ cells, and also in $HR\text{-}30^+$ cells compared to $HR\text{-}30^-$ cells. Protein M2 levels present in the wild type and hydroxyurea-resistant cell lines were shown in Figure 16. The results clearly indicated that protein M2 was dramatically overproduced in the hydroxyurea-resistant $SC2^-$ line and especially in $HR\text{-}30^-$ cell lines compared to the wild type cell line. Furthermore, it was evident from these blots that the level of protein M2 in $SC2^+$ cells was higher than in $SC2^-$ cells. Similarly, the M2 protein levels in $HR\text{-}30^+$ cells was higher than in $HR\text{-}30^-$ cells. All the results agreed with the previous finding by McClarty *et al.* (1987a) and Hurta & Wright (1990a,b).

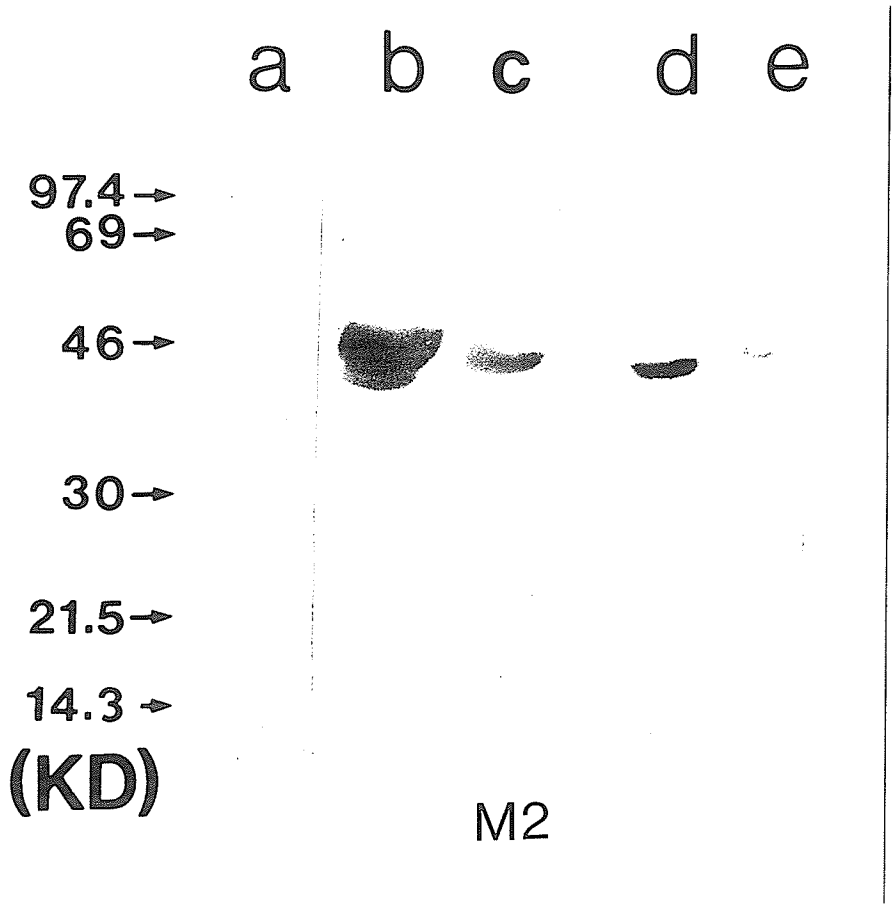
7.0 Growth of Human Carcinoma Cell Lines in the Presence of Hydroxyurea

Human ovarian and colon carcinoma cells have been used widely in studying CDDP-resistant mechanisms (Scanlon *et al.*, 1989). Most of the studies showed a correlation between an elevation in CDDP-targeted molecules (e.g. metallothionein and glutathione) and development of resistance (Kelly and Rozencweig, 1989). In this experiment, we examined the possibility that human CDDP-resistant cell lines would contain alternations in ribonucleotide reductase since the mammalian ribonucleotide reductase was shown to be inhibited by CDDP (Figure 8). We expected that CDDP-resistant cells might be cross-resistant to hydroxyurea if an increase in the enzyme

Figure 16. Western blot analysis for total cellular M2 protein levels in wild type (a), HR-30$^+$ (b), HR-30$^-$ (c), SC2$^+$ (d), and SC2$^-$ (e) cell lines.

Protein preparation and the Western blot procedures were carried out as described under Materials and Methods.

Lane: (a) wild type, 120 μg of protein; (b) HR-30$^+$, 60 μg of protein; (c) HR-30$^-$, 60 μg of protein; (d) SC2$^+$, 20 μg of protein; and (e) SC2$^-$, 20 μg of protein.


a b c d e
97.4
69
46
30
21.5
14.3
(KD)
M2

level is indeed a primary event in the development of CDDP resistance *in vivo*.

The human ovarian carcinoma cell line, A2780S was made resistant to CDDP by weekly 1 hr exposures to 50 μM CDDP for 6 months (Lu *et al.*, 1988) to obtain the cell line called A2780DDP. The human colon carcinoma cell line, HCT8S was also used to isolate a CDDP-resistant cell line named HCT8DDP (Chen and Scanlon, 1988). A2780S, A2780DDP, HCT8S and HCT8DDP cells were grown in RPMI 1640 medium with 10% FBS. The doubling times were 18 hr, 16 hr, 15 hr and 16 hr for A2780S, A2780DDP, HCT8S and HCT8DDP cells, respectively (Table 2). Exposing the cells to 0.2 mM hydroxyurea yielded a doubling time of 31 hr, 28 hr, 25 hr and 21 hr for A2780S, A2780DDP, HCT8S and HCT8DDP cell lines, respectively. Also, when the cells were exposed to 0.3 mM hydroxyurea, the doubling times were 52 hr, 40 hr, 40 hr and 42 hr for A2780S, A2780DDP, HCT8S and HCT8DDP cells, respectively. Therefore, the growth rates of CDDP-resistant cells in the presence of hydroxyurea were approximately the same as CDDP-sensitive cells . This suggests that CDDP-resistant cell lines do not show cross resistance to hydroxyurea, and hence ribonucleotide reductase probably does not involve in the development of CDDP-resistant phenotype in human CDDP-resistant carcinoma cells.

8.0 Colony-forming Ability of Human Carcinoma Cell Lines in the Presence of Hydroxyurea

Table 2. Effects of Hydroxyurea on the Growth Rates of Human Carcinoma Cell lines

Cell lines	Doubling Time (hr)			Ratio *	
	(a)	(b)	(c)	(b)	(c)
A2780S	18	31	52	0.58	0.35
A2780DDP	16	28	40	0.57	0.40
HCT8S	15	25	40	0.60	0.38
HCT8DDP	16	21	42	0.76	0.38

Cells were culturing in RPMI 1640 containing 10% FBS with addition of 0.0 mM (a), 0.20 mM (b) and 0.30 mM (c) of hydroxyurea.

$$^{*}\text{Ratio} = \frac{\text{Doubling time in the absence of the drug}}{\text{Doubling time in the presence of the drug}}$$

To further explore the question that ribonucleotide reductase might play a role for CDDP resistance exhibited in the human CDDP-resistant A2780DDP cells as well as the human colon CDDP-resistant HCT8DDP cells, we measured their colony-forming abilities in the presence of hydroxyurea. The results of a colony-forming study performed at three different concentrations of hydroxyurea are presented in Table 3. Table 3 shows the RPE of A2780S, A2780DDP, HCT8S and HCT8DDP cell lines in the presence of 0.1 mM, 0.2 mM and 0.3 mM of hydroxyurea. Interesting differences in drug sensitivity were observed when A2780DDP cells were compared to parental A2780S cells. The A2780S line was significantly more sensitive to hydroxyurea cytotoxicity than A2780DDP cells ($p < 0.05$ at all hydroxyurea concentrations tested). However, no significant differences in drug sensitivity were observed when HCT8S cells were compared to the CDDP resistant HCT8DDP cell line.

9.0 Levels of Ribonucleotide Reductase in Human Carcinoma Cell Lines

From the knowledge that one of the general CDDP resistant mechanisms is having altered or overexpressed drug targeted molecules (Curt et al., 1984), we expect that CDDP-resistant carcinoma cell lines may have elevated ribonucleotide reductase activity since CDDP can inhibit the activity of ribonucleotide reductase. We

Table 3. Relative Plating Efficiency of Human Carcinoma Cell lines in the Presence of Various Concentrations of Hydroxyurea

Concentrations of Hydroxyurea (mM)	Relative Plating Efficiency ± S.E.* A2780S	A2780DDP	HCT8S	HCT8DDP
0.1	0.83±0.05	0.97±0.08	0.84±0.13	0.75±0.02
0.2	0.28±0.02	0.86±0.08	0.60±0.09	0.58±0.03
0.3	0.10±0.01	0.53±0.04	0.51±0.05	0.41±0.01

* The values presented are the averages of at least 3 independent determinations for 0.1 and 0.2 mM hydroxyurea, and at least 2 independent determinations for 0.3 mM hydroxyurea. Statistically significant differences between the A2780S and A2780DDP cell lines were observed at 0.1 mM hydroxyurea ($p = 0.048$), 0.2 mM hydroxyurea ($p = 0.0001$) and at 0.3 mM hydroxyurea ($p = 0.0001$). No statistically significant differences between HCT8S and HCT8DDP cell lines were detected at any of the three concentrations of hydroxyurea.

were therefore interested in comparing the enzyme activity levels in these CDDP-resistant carcinoma cell lines and their corresponding CDDP-sensitive parental cell lines.

Levels of ribonucleotide reductase activities have been measured in cell preparations obtained from A2780S, A2780DDP, HCT8S and HCT8DDP cells. Table 4 shows that enzyme prepared from A2780S, A2780DDP, HCT8S and HCT8DDP cell lines had specific CDP reductase activities of 0.09, 0.14, 0.20 and 0.22 nmole dCDP/hr/mg protein, respectively. In keeping with the relationship between hydroxyurea sensitivity and enzyme activity (Wright, 1987, 1989a & 1990), the A2780DDP line exhibited about 56% elevation in the level of ribonucleotide reductase activity compared to that of the parental CDDP-sensitive A2780S cells. In addition, as expected from hydroxyurea sensitivity studies, the HCT8S and HCT8DDP cell lines contained approximately the same levels of ribonucleotide reductase activity.

10.0 Ribonucleotide Reductase Message Levels in Human Carcinoma Cell Lines

It is now established from studies of hydroxyurea resistant cell lines that changes in resistance to this drug are accompanied by changes in ribonucleotide reductase activity and gene expression (Wright, 1989a and 1990). All hydroxyurea resistant cell lines described to date exhibit elevations in the expression of the M2

Table 4. Levels of Ribonucleotide Reductase Activity in Human Carcinoma Cell Lines

Cell Lines	Specific Enzyme Activity (nmoles dCDP/hr/mg protein) ± S.E.*
A2780S	0.087±0.021
A2780DDP	0.135±0.014
HCT8S	0.201±0.013
HCT8DDP	0.219±0.016

* The values presented are the average of at least 3 independent determinations ± S.E.. Statistical analysis indicated a trend toward significantly higher levels of enzyme activity in A2780DDP cells when compared to parental A2780S cells ($p = 0.067$). No statistically significant differences were found when enzyme levels in HCT8S cells were compared to levels in HCT8DDP cells.

gene, with or without changes in M1 gene expression (Wright, 1987, 1989a & 1990). The M2 component is limiting for enzyme activity, as an increase in this component alone can lead to an elevation in ribonucleotide reductase activity (Wright, 1987, 1989a & 1990). This experiment was carried out to examine the cellular M1 and M2 mRNA levels present in the two CDDP-resistant and the two CDDP-sensitive carcinoma cell lines. Northern blot analysis using M1 cDNA is presented in Figure 17. These results are consistent with other investigations of mammalian cell lines, which showed the presence of a single M1 mRNA species of about 3.1 kilobases (Kb) (Thelander & Berg, 1986; Wright et al., 1987). Interestingly, M2 cDNA-probed Northern blots, presented in Figure 18, revealed two M2 mRNA species of different molecular weights in all the cell lines. The high-molecular-weight species was about 3.4 kb and the low-molecular-weight mRNA was approximately 1.6 kb. These results do agree with previous observations from Northern blot analysis of M2 mRNA levels in Chinese hamster ovary cells, rat L_6 myoblast cells as well as human Hela S_3 cells (Wright *et al.*, 1987).

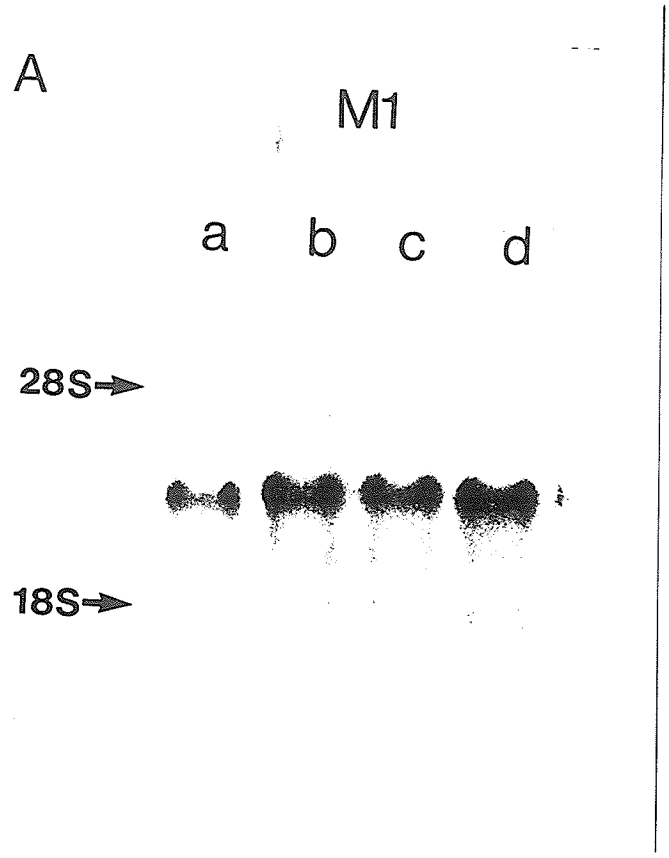
Results from Northern blot analysis of M1 mRNA level (Figure 17) and M2 mRNA level (Figure 18) were quantified by densitometric scanning. RNA loading was checked by comparing the message levels of the control gene, GAPDH in each lane (Choy *et al.*, 1989) (Figure 17B and 18B). Consistent with the hydroxyurea sensitivity studies (Table 3) and the enzyme activity results (Table 4), we observed that the A2780DDP line contained increased levels of M2 mRNA. Densitometric analysis indicated approximately a 2.1 fold elevation in M2 message when A2780DDP cells

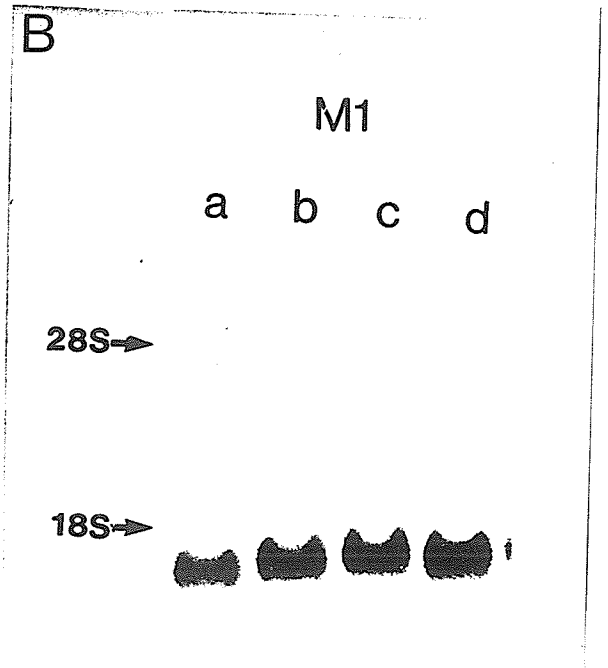
Figure 17. Northern blots of M1 mRNA in the cisplatin-sensitive and -resistant cell lines. For Northern blots, 20 μg of total cellular RNA isolated from A2780S, A2780DDP, HCT8S and HCT8DDP cells were denatured and run on 1% agarose formaldehyde gels as indicated in Materials and Methods.

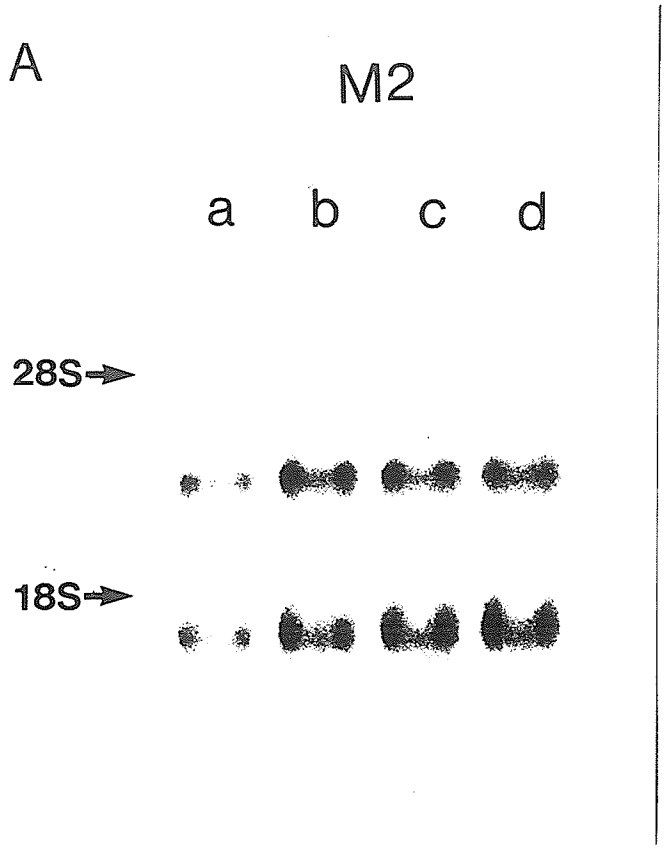
(A). Northern blot of M1 mRNA: (a) A2780S, (b) A2780DDP, (c) HCT8S and (d) HCT8DDP.

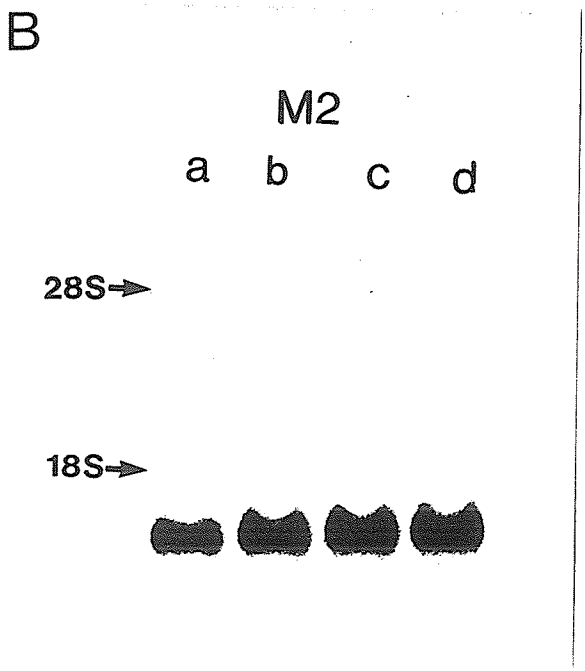
(B). Equal loading of RNA was determined by reprobing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as described (Choy *et al.*, 1989).

The position of 28s and 18s rRNA are indicated. Autoradiogram were exposed for 4 days (A) or 24 hr (B) at -70°C with intensifying screens.


A
M1
a
b
c
d
28S
18S


B
M1
a
b
c
d
28S
18S


A
M2
a b c d
28S
18S


B
M2
a b c d
28S
18S

were compared to the parental A2780S line. No significant changes in the M1 mRNA levels were detected. Also, as expected from the hydroxyurea sensitivity and enzyme activity results obtained with the HCT8S and HCT8DDP cell lines, no differences were found in M1 or M2 mRNA levels when these two cell lines were compared.

DISCUSSION

1.0 Effects of CDDP on Mammalian Ribonucleotide Reductase

Cisplatin is one of the most widely used cancer chemotherapeutic agents. It has been used in treatment of several malignancies, alone or in combination with other anticancer drugs (Rosenberg, 1985). The mode of action by which the CDDP stops tumor growth is still unclear. Studies to date have proposed several different cytotoxic mechanisms, such as, direct interaction of CDDP with DNA molecules resulting in inhibition of DNA synthesis; formation of a chelate complex with key enzymes containing redox-active thiol groups; and activation of host defense mechanisms (Roberts & Thomson, 1979; Rosenberg, 1985). Whether one or all of these mechanisms cause the killing of tumor cells, is still waiting to be conclusively demonstrated. However, the most widely accepted view is that the primary mechanism by which CDDP prevents tumor growth, is through the formation of intrastrand crosslinks with DNA and inhibition of DNA synthesis.

Although the binding of CDDP to DNA has been studied in detail (Sherman & Lippard, 1987), a number of features concerning the *in vivo* activity of CDDP remain to be clarified. For example, cis-dichloroethylenediamine platinum (II) with T7 bacteriophage DNA, at the drug dose required to reduce phage activity to 37% of control values (i.e. its mean lethal dose) only bound five Pt-derived molecules to the DNA (Shooter *et al.*, 1972; Pinto & Lippard, 1985). Moreover, many studies also reported that CDDP specifically inhibited some enzymes containing SH-groups, which contributed to the cytotoxic side effects of the drug in patients (Levi *et al.*, 1980;

Nechay & Neldon, 1984).

Ribonucleotide reductase participates in a major pathway involved in the synthesis of DNA, thereby playing a key regulatory role in DNA synthesis in all eucaryotic and procaryotic cells. *E.coli* ribonucleotide reductase has recently been shown to be inhibited by CDDP (Smith & Douglas, 1989). *E.coli* ribonucleotide reductase shares many similar structural properties with mammalian and virus coded ribonucleotide reductases; therefore, the observation from the Smith and Douglas study (1989) noted above prompted us to investigate the effects of CDDP on mammalian ribonucleotide reductase.

The effects of CDDP on mammalian ribonucleotide reductase were studied using a wild type mouse L cell line (L60) and its hydroxyurea-resistant mutant cell lines. The mouse L cell lines were used because a series of hydroxyurea-resistant mutant cell lines has previously been isolated (Choy *et al.*, 1988). These hydroxyurea-resistant cell lines exhibited an elevation in ribonucleotide reductase activity and provided a useful system to examine the effects of CDDP on these ribonucleotide reductase overproducing cell lines. SC2 and HR-30 cell lines are two of these mutant cell lines selected for resistance to 5 mM and 30 mM hydroxyurea, respectively. SC2 and HR-30 have been shown to have an elevation in both M1 and M2 subunits (Choy *et al.*, 1988; Hurta & Wright, 1990a,b).

In this study, mammalian ribonucleotide reductase was shown to be inhibited by CDDP *in vitro* (Figure 8). 50% enzyme inhibition occurred at about 0.31 mM CDDP with enzyme prepared from L60 mouse wild type cells. This agrees with the

results in the Smith & Douglas (1989) study (50% inhibition occurred at about 0.4 mM for *E.coli* ribonucleotide reductase). Given that ribonucleotide reductase occupies such a crucial metabolic position, that CDDP was found to specifically inhibit DNA synthesis rather than RNA or protein synthesis (Roberts & Thomson, 1979), and that CDDP has a great affinity for polarizable ligands (such as -SH group), we hypothesized that cisplatin inhibition of ribonucleotide reductase activity *in vivo* may provide a secondary lethal site of action for this drug.

2.0 Differences in *E.coli* and Mammalian Ribonucleotide Reductase Responses to CDDP

In the Smith & Douglas (1989) study, the *E.coli* ribonucleotide reductase was demonstrated to be more sensitive to CDDP inhibition under anaerobically enriched conditions, and it was suggested that this was because the reduced-dithiol form was more reactive to CDDP. In addition, the anaerobically enriched condition is more similar to the physiological situation, in which ribonucleotide reductase is maintained in reduced form by several reduction systems (Figure 6). We therefore, carried out the enzyme inhibition assay under N_2 enriched conditions in order to minimize the oxidation of dithiol groups. The presence of DTT in the assay mix (see Materials and Methods) also helped to keep ribonucleotide reductase in a reduced form. Results shown in Figure 10 indicate that there was no difference between the mammalian

enzyme inhibition observed under anaerobically enriched conditions and under aerobic conditions. These observations are unlike those obtained in the *E.coli* study (Smith & Douglas, 1989). This indicates some differences exist between *E.coli* and mammalian ribonucleotide reductase, although they exhibit similar enzyme activity and allosteric regulation (Wright, 1983; 1989). There also exists some other evidence to indicate important differences in structure and catalytic mechanisms between the bacterial and mammalian enzymes. For example: the EPR (electron paramagnetic resonance) spectrum which results from the presence of a B2 or M2 tyrosyl free radical is significantly different (Graslund *et al.*, 1982; Sahlin *et al.*, 1987); inhibition of mammalian M2 protein with hydroxyurea or 2'-azidoCDP is reversible, in contrast to the irreversible inhibition of the B2 subunit of the E.coli reductase, hence suggesting a different catalytic process (Engstrom *et al.*, 1979); thiosemicarbazone inhibits reductase from Novikoff hepatoma to 50% at about 0.1 μM, while no inhibition of the *E.coli* enzyme was found at this concentration (Moore, *et al.*, 1970); and some studies (Caras *et al.*, 1985; Thelander & Berg, 1986) on M1/B1 cDNA and M2/B2 cDNA sequences revealed only about 37% homologies in amino acid sequences between mouse M1 and *E.coli* B1 subunits, and even lower sequence homology was observed between mouse M2 and *E.coli* B2 subunit.

In the case of *E. coli* ribonucleotide reductase, the site of inhibition by CDDP was found to be the B1 subunit (Smith & Douglas, 1989). The B1 subunit, a 175 kd dimer of similar polypeptides, contains substrate binding sites, allosteric regulatory sites, and a number redox-active thiols (Stubbe, 1990). Two pairs of redox-active

thiols are involved in enzyme activity, Cys-752/Cys-757 and Cys-222/Cys-227 (Lin *et al.*, 1987). The Cys-222/Cys-227 dithiol functions as an electron acceptor from thioredoxin and shuttles reducing equivalents into the active-site thiols *via* disulphide interchange, and the C-terminal dithiol Cys-752/Cys-757 is directly responsible for nucleoside diphosphate reduction . The structure of mammalian ribonucleotide reductase is not as well characterized as that of *E. coli.* There is 37% homology between mouse M1 and *E.coli* B1 observed from cDNA sequences (Caras *et al.*, 1985). The M1 subunits also contain two pairs of active site dithiol groups, which should be the potential site of action of CDDP (Wright *et al.*, 1990).

To further elucidate the mechanism of drug action, we separated the M1 protein from M2 protein by blue dextran-sepharose affinity chromatography to study the action of CDDP on M1 and M2 individually. As shown in Figure 11A,B, M1 and M2 were successfully separated from each other, as demonstrated by the lack of enzyme activity with M1 or M2 alone. The effect of CDDP on each individual subunit, M1 or M2, was examined by incubating an increasing amount of CDDP with the M1 or M2 subunit, and then assaying ribonucleotide reductase activity individually by adding untreated complementary subunit. Unreacted CDDP was removed from the inhibition mixture by G-25 sephadex gel filtration, so that no free CDDP could react with the other ribonucleotide reductase component during the enzyme assay. Results from the study indicate that inhibition of ribonucleotide reductase by CDDP occurs through the interaction of the drug with both the M1 and the M2 subunits, which as noted earlier, is unlike the observations obtained in the *E. coli* study (Smith

and Douglas, 1989). These results suggest that there are significant differences between the structure of the B2 and M2 subunits. When the amino acid sequence of the B2 subunit is compared with that of the M2 subunit, there is a very low homology, and therefore, CDDP can interact with the B2 and M2 subunits differently. Although the enzyme inhibition by CDDP in *E.coli* was due to inhibition of the B1 subunit exclusively, it is not surprising to observe the involvement of the M2 subunit in the inhibition of mammalian ribonucleotide reductase, since there are five cysteine amino acid residues (Cys-161, -203, -271, -275, and -218) present in the M2 subunit (Thelander & Berg, 1986) and Cys-161 is quite close to the active-site free radical tyrosyl residue (Tyr-177). However, the relative position between cysteine residues and the active site in the 3-D structure of the functional protein is not precisely known. It seems possible that CDDP may be interacting with the Cys-161 residue and affecting free radical regeneration that is required for enzyme activity. Also, it is possible that CDDP interacts with any one or several of the other cysteine residues of the M2 subunits, and hence causes the inhibition of enzyme activity either through conformational changes to the protein, or perhaps through blocking binding or releasing of substrates or products. It is interesting to note that the *E.coli* B2 subunit does not have an equivalent cysteine residue close to the tyrosyl free radical, which could perhaps explain why the B2 subunit is not a target for CDDP inhibition. Further studies are required to answer this interesting question.

3.0 Hydroxyurea and CDDP Resistant Mutants as Tools to Determine the Importance of Ribonucleotide Reductase as a Target for CDDP

SC2 and HR-30 mutant cell lines have different levels of increment in M1 and M2 protein levels. It has been previously reported that SC2 mutant cells growing in the absence of 5 mM hydroxyurea showed a 2-3 fold elevation in M1 protein level and a 50 fold elevation in M2 protein level. Also, it was found that when the SC2 and HR-30 cell lines were grown in the presence of hydroxyurea, they showed a further increase in the ribonucleotide reductase activity, which was accompanied by an elevation in the levels of M1 and M2 subunits (McClarty *et al.*, 1987b; Hurta & Wright, 1990b). The HR-30 mutant cell line growing in the absence of hydroxyurea, resulted in a 15-20 fold and 100 fold elevation in M1 and M2 protein levels, respectively, (Hurta & Wright, 1990b). In the present study, Western blot analysis has been used to measure the cellular levels of M1 and M2 protein in the wild type mouse L cell (L60) and the two hydroxyurea-resistant cell lines ($SC2^{-/+}$ and $HR\text{-}30^{-/+}$) (Figure 15 and 16). In agreement with the previously published results, the following points are concluded from the observations obtained in Western blot studies: (1) hydroxyurea-resistant cell lines, $SC2^{-}$ and $HR\text{-}30^{-}$, had an elevation in both M1 and M2 protein levels compared to that of wild type cell line; (2) growing hydroxyurea-resistant cells in culture medium containing 5 mM hydroxyurea, (i.e. $SC2^{+}$ and $HR\text{-}30^{+}$ cells) led to a further elevation in both M1 and M2 protein levels; (3) the

elevation of M2 protein levels in HR-30$^+$ cells was more dramatic than its corresponding M1 protein level change.

Since, SC2$^-$, SC2$^+$, HR-30$^-$ and HR-30$^+$ cell lines contain different ratios of M1 to M2 (Table 1), and since the two subunits show different sensitivities to CDDP inactivation, we would expect some variation in enzyme sensitivity to CDDP depending upon which cell line was used to obtain enzyme preparation. The results from enzyme inhibition studies confirmed this prediction (Figure 9). For example, enzyme prepared from HR-30$^-$ cells (M1/M2 = 0.18) contained about a 2.6 fold higher M1/M2 ratio than that of SC2$^-$ cells (M1/M2 = 0.05). Also enzyme prepared from HR-30$^-$ cells required about 2.5 fold higher CDDP concentration to produce a 50% enzyme inhibition than enzyme prepared from SC2$^-$ cells, suggesting that extracts with a higher M1/M2 ratio were more resistant to the inhibitory effects of the drug.

The cytotoxic effects of CDDP in cultured cells have also been examined in this study in an attempt to correlate enzyme inhibition *in vitro* to cytotoxicity of the drug in cultured cells. The wild type mouse L60 cell line, and hydroxyurea-resistant mutant cell lines, SC2 and HR-30 were used in these experiments. The cytotoxic effects of the drug on these cell lines were examined by measuring their colony-forming abilities when growing in the presence of various CDDP concentrations. The concentration required to inhibit colony formation was approximately 1.0 μM CDDP, whereas concentrations beginning at about 100 μM of the drug inhibited the enzyme activity. This suggests that ribonucleotide reductase is probably not the primary target molecule for CDDP cytotoxicity *in vivo*. However, it may play a secondary role,

particularly if the cellular concentrations of CDDP vary from one intracellular location to another. This would be dependent upon the possibility that CDDP is concentrated to high levels in regions of the cell where ribonucleotide reductase is also located. Further work is required to test this hypothesis. By comparing the colony-forming abilities of wild type and hydroxyurea-resistant cell lines, it was clear that there was no dramatic difference in terms of the RPE in the presence of CDDP (Figure 13). Again, this result also supports the above suggestion, because if ribonucleotide reductase is a primary site of action for CDDP in cultured cells, the highly hydroxyurea-resistant cell lines, HR-30$^-$ and HR-30$^+$ should then exhibit significantly higher resistance towards the drug than the wild type cell. This is because the drug resistant lines have elevated levels of M1 and M2 proteins and increased enzyme activity. However, in Figure 14, only the cell line HR-30$^+$ showed a slightly higher resistance to CDDP when compared to wild type cells. This may be due to the differences between HR-30 and L60 cell lines in terms of (i) permeability of the cell membrane to the CDDP, (ii) efficiency of the DNA repair system, and (iii) levels of a variety of SH-containing CDDP-target proteins, including perhaps, ribonucleotide reductase.

One of the obstacles in cancer chemotherapy is the establishment of drug resistance as well as the development of cross resistance to other drugs (Vickers *et al.*, 1989; Fojo, 1991). After an initially favourable response, CDDP-resistant tumor cell populations frequently develop (Rosenberg, 1985, Ozols & Young, 1984b; Scanlon & Kashani-Sebet, 1989c). Furthermore, CDDP-resistant cells were reported

to be cross-resistant to other chemotherapeutic agents, such as methotrexate and 5-fluorouracil in a number of tumors (Schabel *et al.*, 1983; Teicher *et al.*, 1987; Newman *et al.*, 1988). The mechanisms of resistance to CDDP is still complicated and controversial. Multiple mechanisms of drug resistance have been suggested in CDDP-resistant animal and human tumor models, which have already been discussed in the Introduction section.

The CDDP-resistant mechanism in human cultured carcinoma cells has been studied in detail by Scanlon's group and by other investigators (reviewed by Kelly & Rozeneweig, 1989). A number of biochemical and molecular properties have been altered in the CDDP-resistant human ovarian carcinoma cell line, A2780DDP, and the CDDP-resistant human colon carcinoma cell line, HCT8DDP (Chen & Scanlon, 1988; Lu & Scanlon, 1988; Scanlon *et al.*, 1989b). Observations from previous studies of these lines can be summarized in the following comments: (1) enhanced DNA repair (Musuda *et al.*, 1988, 1990); (2) elevation of DNA polymerase [5 fold and 4 fold increases in DNA polymerase ß mRNAs were observed in A2780DDP and HCT8DDP cell lines, respectively (Scanlon *et al.*, 1989c)]; (3) elevation of GSH level (Hamilton *et al.*, 1985); (4) enhanced folate metabolism [both A2780DDP and HCT8DDP displayed 3-5 fold elevated mRNA for dTMP synthase and thymidine kinase (Lu *et al.*, 1988; Scanlon & Kashani-Sabet, 1988; Chen & Scanlon, 1988)]; (5) enhanced expression of oncogenes [both A2780DDP and HCT8DDP expressed 4 fold higher levels of c-*fos* and H-*ras* oncogenes (Chen & Scanlon, 1988; Lu *et al.*, 1988)], and in A2780DDP cells, mRNA for c-*myc* increased 3-5 fold, but no change was

observed with HCT8DDP cells (Scanlon *et al.*, 1989b). All of these observations might be important in the development of the drug resistant phenotypes exhibited by A2780DDP and HCT8DDP mutant cell lines.

Since ribonucleotide reductase was observed to be inhibited by CDDP, we were therefore interested in examining the possibility that ribonucleotide reductase may play a role in the drug resistant phenotype of the CDDP-resistant cell lines. First, the growth rates of CDDP-resistant cell lines, A2780DDP and HCT8DDP in the presence of hydroxyurea, were compared to their CDDP-sensitive parental cell lines, A2780S and HCT8S. In the presence of 0.2 mM and 0.3 mM of hydroxyurea, both CDDP-sensitive and -resistant cells exhibited approximately a 2 fold increase in their doubling times (Table 2). At the concentration of 0.2 mM, the ratio of the doubling time in the absence of the drug to that in the presence of the drug was not obviously different between the CDDP-sensitive and -resistant cells. A similar response was observed at the concentration of 0.3 mM hydroxyurea. Secondly, the cytotoxic effects of the hydroxyurea on these CDDP-resistant cell lines were examined by measuring their colony-forming abilities in the presence of the drug. The results shown in Table 3 suggest that hydroxyurea has the same cytotoxic action with the CDDP-sensitive HCT8S and the CDDP-resistant HCT8DDP cell lines. However, CDDP-resistant A2780DDP cells showed more resistance to hydroxyurea when compared to its CDDP-sensitive parental A2780S cell line as the concentration of hydroxyurea increased. Thirdly, the levels of ribonucleotide reductase activity in the CDDP-resistant cells were compared to their CDDP-sensitive parental cells. HCT8S

and HCT8DDP exhibited approximately the same level of ribonucleotide reductase activity (Table 4). Interestingly, the A2780DDP line had a 1.5 fold increase in ribonucleotide reductase activity compared to the parental A2780S cells. Lastly, the mRNA levels of two ribonucleotide reductase subunits, M1 and M2 in these CDDP-resistant cells were examined by Northern blotting studies using M1 and M2 cDNA. Densitometric analysis has been performed on both M1 and M2 cDNA-probed Northern blots (Figure 17 and 18) to quantify the total cellular M1 and M2 mRNA levels. Results indicated that the HCT8S and HCTDDP lines had the same level of M1 and M2 mRNAs. The A2780DDP cell line however, had an average 2.3 fold elevation in the M2 mRNA level (when the two M2 mRNA species were analyzed) without an obvious change in M1 mRNA level, when compared to parental A2780S cells. From these four experimental approaches, it is interesting to note that (i) the CDDP-resistant ovarian carcinoma A2780DDP cell line showed less sensitivity to the cytotoxic effect of hydroxyurea in colony forming experiments when compared to its CDDP-sensitive parental A2780S cell line; (ii) the A2780DDP cell line exhibited a modest elevation in cellular ribonucleotide reductase activity; and (iii) the A2780DDP cell line had an elevation in M2 mRNA level. Whether the observation that the CDDP-resistant A2780DDP cell line behaves differently from the CDDP-resistant HCT8DDP cell line is due to differences in CDDP resistance mechanisms or is simply due to unrelated cell line variation, is unknown. More investigations are required to examine this point. However, when all the results are considered, it seems likely that ribonucleotide reductase does not play a major role in the establishment of CDDP-

resistance.

Conclusion: this is the first time that mammalian ribonucleotide reductase has been shown to be inhibited by the anticancer drug, CDDP. The inhibition of the mammalian ribonucleotide reductase activity has been shown to be brought about by CDDP interaction with the M1 and M2 subunits of the enzyme. The mechanism of the drug action is thought to be through the formation of a chelate complex between CDDP and M1 and/or M2 subunit *via* the dithiol groups. By viewing the cytotoxicity of CDDP exhibited in mouse hydroxyurea-resistant cell lines and the cytotoxicity of hydroxyurea exhibited in CDDP-resistant cell lines, it seems ribonucleotide reductase is not a primary target for CDDP. However, it is still possible that mammalian ribonucleotide reductase may play a secondary role in the lethal activity of the drug in cancer chemotherapy and in CDDP resistant mechanisms, at least in some situations.

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