enzymatically negative were re-analysed with the same result and the two enzymatic assays gave corresponding results as shown in Fig. 3. Interestingly, two individuals had activities at the upper end of the low activity scale, especially with the 50 μM TSO assay designed to differentiate within the individuals of low activity. Thus, these three discrepant cases are probably not due to experimental error, but rather may indicate that conjugation activity may become phenotypically low e.g. due to disease or unknown environmental factors. Moreover, we cannot exclude rare mutations located at other sites of the gene that may not be detected by the PCR methods employed here.

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Carboplatin as opposed to cisplatin does not stimulate the expression of the human immunodeficiency virus long terminal repeat sequences

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Abstract—The recombinant plasmid pBHIV1 carrying the long terminal repeat (LTR) of the human immunodeficiency virus 1 (HIV-1), linked to the chloramphenicol acetyl transferase (CAT) gene, was introduced into human and rat fibroblasts. Stable transfectants resistant to geneticin expressed CAT activity from the HIV-1 LTR. It was found that the cytotoxic drug *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin) at concentrations from 1×10^{-6} to 1×10^{-4} M does not stimulate the expression of CAT from the HIV-1 LTR. These results differ from previous studies with the related drug *cis*-diamminedichloroplatinum(II) which showed stimulation of gene expression from the HIV-1 LTR and suggest that carboplatin could be used in the treatment of cancer patients with Acquired Immune Deficiency Syndrome.

cis-Diamminedichloroplatinum(II) (cisplatin*) is an important cytotoxic agent used in the chemotherapy of several types of human tumor such as testicular, ovarian and lung cancer [1]. However, the severe toxicity related to this compound led to the development of second generation analogues [2]. Of these, carboplatin lacks much of the

* Abbreviations: LTR, long terminal repeat; HIV-1, human immunodeficiency virus 1; CAT, chloramphenicol acetyl transferase; carboplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum (II); cisplatin, *cis*-diammine dichloroplatinum(II); FCS, fetal calf serum.

renal toxicity, neurotoxicity and ototoxicity of the parent compound but is myelotoxic [3].

The therapeutic effectiveness of carboplatin and cisplatin is similar even though dose-limiting toxicities of these drugs are different [4, 5].

The molecular pharmacology of these agents is complex; however, it is generally accepted that the cytotoxic lesion is formed by hydration of platinum which then binds to the N_7 residue of guanine causing inter- and intra-strand DNA cross-links. Cisplatin and carboplatin are hydrated to a bihydroxylated platinum intermediate which binds to DNA, but the rate of this reaction is much slower for carboplatin than for cisplatin [6]. The drugs also have different pharmacokinetics and distributions within the body and this could account for their different patterns of toxicity [7].

HIV-1 is the etiological agent of the Acquired Immune Deficiency Syndrome [8]. The syndrome is associated with a range of malignancies including Kaposi's sarcoma, non-Hodgkin's lymphoma, squamous cell carcinoma, testicular cancers, malignant melanoma, primary hepatocellular carcinoma and Hodgkin's disease [9].

The HIV-1 LTR has a complex structure comprised of protein binding sites which control reactivation of latent virus leading to further cycles of infection. A number of signals act on the 5'LTR to induce viral mRNA transcription, including the HIV *trans*-activator *tat* [10, 11], heterologous *trans*-activators from other viruses (e.g. Herpes Simplex virus, cytomegalovirus [12], positive regulatory factors (e.g., NF-kB) induced by cell activation [13, 14] and the oncogene *ras* [15]. In a previous study we have found that cisplatin stimulates the expression of the reporter CAT gene from the HIV-1 LTR in rat and human fibroblasts respectively [16, 17]. In the present study we have examined the effect of carboplatin on the HIV-1 LTR-driven expression of cat in rat and human fibroblasts, and found that carboplatin does not stimulate transcriptional activation of CAT in these systems.

Materials and Methods

Recombinant plasmids and cell lines. Plasmid pBHIV1 carrying a 728 bp *Xho*I-*Hind*III DNA fragment containing the HIV-1 LTR sequences was constructed by inserting a 1.9 kb *Bam*HI fragment carrying the *aph* gene into the single *Bam*H1 site of plasmid pBC12/HIV/CAT [18].

The spontaneously immortalized rat 208F and the SV40 immortalized human MRCSV40TGR fibroblasts were used as recipients to obtain the RFBHIV1-1 and SVTGHIV-1 stable geneticin-resistant transfectants with plasmid pBHIV1 [16]. DNA transfections were carried out using the calcium phosphate technique [19] as modified previously [20].

CAT assays. Cells were grown exponentially in Ham's medium containing 10% FCS and assayed for activity as described previously [21].

Assay for cell proliferation. The rapid colorimetric assay for cell proliferation of Mosmann [22] was used. This assay has been described in detail previously [16]. Briefly, a stock solution of 3-(4,5-dimethylthiazol-2,5-diphenyl tetrozolium bromide (from the Sigma Chemical Co., Poole, U.K.) in phosphate-buffered saline (5 mg/mL, filter-sterilized) was prepared. This was added to each well (10 μL per 100 μL medium) and plates were incubated at 37° for each time interval. 0.04 NHCI (110 μL) in isopropanol was added to each well and after thorough mixing (to dissolve the dark blue crystals) the plates were left for a few minutes at room temperature. Then the plates were placed on a Titertek Flow MicroELIZA reader and optical density was recorded at the wavelength of 540 nm. Plates were read within 1 hr of adding the acid isopropanol solution.

Results

Cisplatin enhances transcription from the HIV LTR sequences. The transfectant RFBHIV1-1 cells were treated with cisplatin (5×10^{-5} M) at various time intervals. A representative CAT assay is shown in Fig. 1a and the corresponding histogram in Fig. 1b. Optimal stimulation was obtained at 24 hr when CAT activity increased 34-fold. Similar results were also obtained with the human SVTGHIV1-1 cells treated with cisplatin as described previously [17].

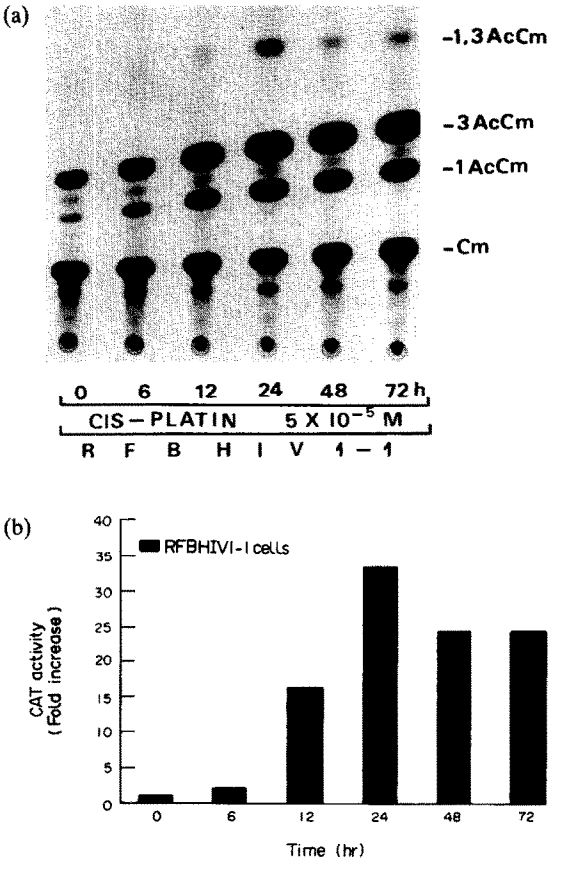

Fig. 1. Induction of CAT activity in RFBHIV1-1 cells by cisplatin at various times post treatment. (a) Chromatogram for representative CAT assays with extracts from RFBHIV1-1 cells treated with 5×10^{-5} M cisplatin at various times. (b) Histogram of recorded CAT activities. RFBHIV1-1 cells were plated at 1.5×10^6/75 cm² flask in Ham's SF12 containing 10% FCS at 37°. The medium was replaced 24 hr later with Ham's SF12 containing 0.5% FCS and left for another 24 hr at 37°. Then the medium was changed with Ham's SF12 containing 5% FCS and 5×10^{-5} M of cisplatin was added. Cells were harvested 0, 6, 12, 24, 48 and 72 hr later and tested for CAT activity. The relative value of CAT activity in RFBHIV1-1 was 1.3 nmol acetylated chloramphenicol/μg protein/hr incubation. Values are means from three experiments; SD was less than 3% of the mean values.

Carboplatin does not stimulate transcription from the HIV-LTR sequences. The recipient rat 208F and human MRCSV40TGR, and their respective derivative RFBHIV1-1 and SVTGHIV1-1 transfectant cell lines were treated with carboplatin at concentrations between 1×10^{-6}–1×10^{-4} M. A representative CAT assay is shown in Fig. 2a and the corresponding histogram in Fig. 2b.

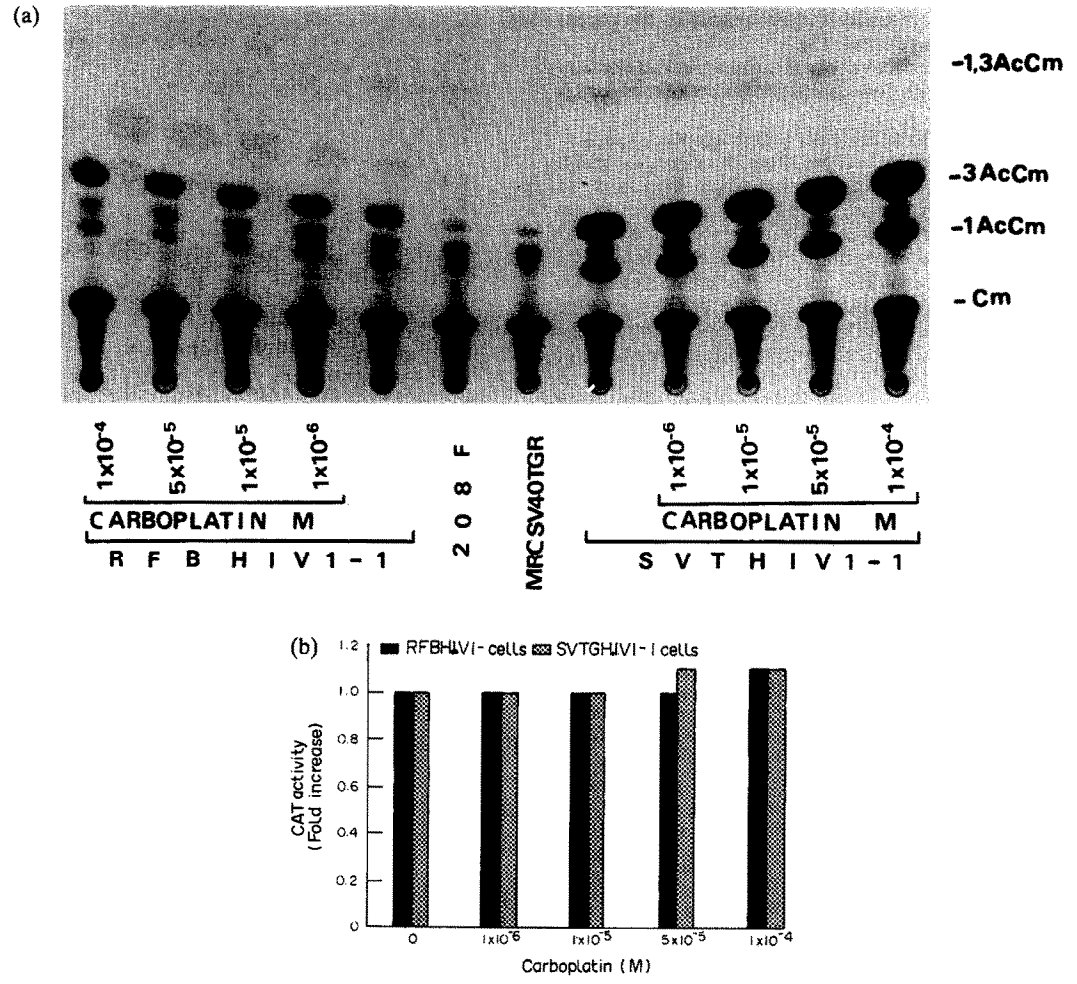

Fig. 2. Induction of CAT activity by carboplatin. (a) Chromatogram of representative CAT assays with extracts from recipients 208F and MRCSV40TGR, and transfectants RFBHIV1-1 and SVTGHIV1-1 cells with and without treatment with carboplatin. (b) CAT values were computed as described in Fig. 1 and are presented in histograms. Relative values of CAT activity in RFBHIV1-1 and SVTGHIV1-1 were 1.2 and 34 nmol acetylated chloramphenicol/μg protein/hr incubation, respectively. The length of exposure time to carboplatin was 24 hr. Values are means from three experiments; SD was less than 3% of the average values.

At the optimal cisplatin concentration of 5×10^{-5} M, (see Refs 16 and 17) a 22-fold increase in CAT activity was observed while in carboplatin-treated cells no corresponding stimulation was obtained.

Carboplatin toxicity. The cytotoxic effect of carboplatin on RFBHIV1-1 and SVTGHIV1-1 cells was measured by a rapid cell proliferation assay, for different time exposures (0, 24, 48, 72, 96 and 120 hr) over a range of carboplatin concentrations (1×10^{-6}–1×10^{-4} M). As shown in Fig. 3a and b, carboplatin inhibited cell proliferation but was less effective than cisplatin [16, 17].

Discussion

HIV-1 gene expression and replication is tightly controlled through the interaction of *trans*-acting regulatory proteins (Tat, Rev, Nef) and host cell factors with *cis*-acting elements (TAR, PRE, NRE) present in viral DNA and RNA [10, 23, 24]. The HIV-1 LTR plays an important role in viral behaviour in the host cell as it carries *cis*- or *trans*-acting sequences responding to cellular [15, 25] or viral gene products [11, 23, 24].

In a previous study we have examined the effect of cisplatin on the transcriptional activation of the HIV-1 LTR employing the two stable transfectant cell lines RFBHIV-1 and SVTGHIV1-1 of rat and human origin, respectively, expressing the reporter CAT gene from the HIV-1 LTR sequences. We have found that in rat and human fibroblasts, cisplatin at the optimum concentration of 5×10^{-5} M, induces CAT activity 22- and 2.2-fold, respectively [16, 17]. In the present study we have investigated whether carboplatin could exert a similar effect. It was found that the HIV-1 LTR promoter does not respond to carboplatin (Fig. 2a and b). Despite the fact that the active, molecular principle is the same for cisplatin and carboplatin, there is a clear difference in their ability to interact with the HIV-1 LTR promoter. This could be explained partly by the difference in the kinetics of hydration of the two compounds which has been shown to affect the rate of binding to DNA (slower for carboplatin)

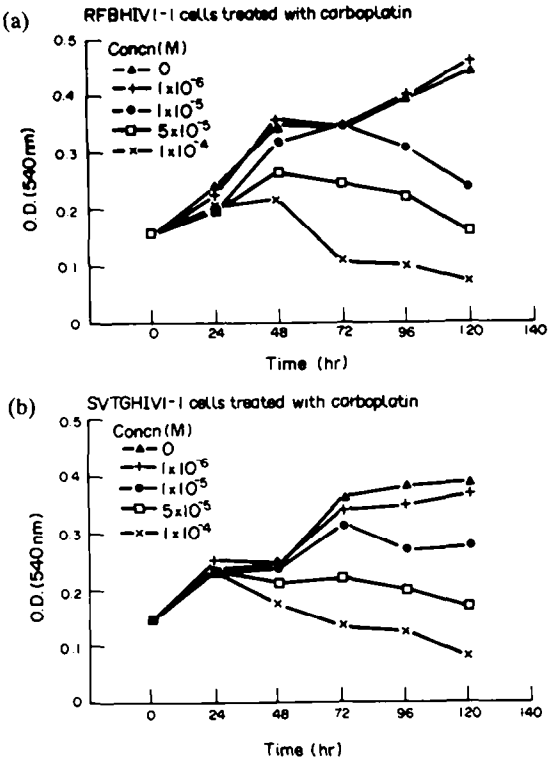

Fig. 3. Cell proliferation in response to carboplatin after various exposure times. Exponentially growing (a) RFBHIV1-1 and (b) SVTGHIV1-1 cells (4×10^{-3}) were plated in 96-well tissue culture clusters (costar) in Ham's SF12 medium containing 10% FCS in the presence of the indicated concentration of carboplatin. At the indicated times cell proliferation was measured using Mosmann's rapid colorimetric assay.

[6]. This probably accounts also for the fact that a greater concentration of carboplatin than cisplatin is required to kill the transfectant cell lines, i.e. the cytotoxic dose–response curve for carboplatin is shifted to the right. This implies that carboplatin can kill the transfectant cells without activating the HIV-1 promoter.

Recent evidence from our laboratory indicated that cisplatin binds to the negative regulatory element (NRE) of the HIV LTR. We plan to synthesize oligonucleotides corresponding to this region and determine the binding and specific adduct formation following exposure to cisplatin and carboplatin in an attempt to explain the differential cell behaviour of these drugs.

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Generation of free radicals during the reductive metabolism of nilutamide by lung microsomes: possible role in the development of lung lesions in patients treated with this anti-androgen

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Abstract—The pulmonary metabolism of nilutamide, a nitroaromatic anti-androgen drug leading to pulmonary lesions in a few recipients, has been investigated in rats. Incubation of nilutamide (1 mM) with rat lung microsomes and NADPH under anaerobic conditions led to the formation of the nitro anion free radical, as indicated by ESR spectroscopy. The steady state concentration of this radical was not decreased by CO or SKF 525-A (two inhibitors of cytochrome P450), but was decreased by $NADP^+$ (10 mM) or *p*-chloromercuribenzoate (0.47 mM) (two inhibitors of NADPH-cytochrome P450 reductase activity). Anaerobic incubations of [^{3}H]nilutamide (0.1 mM) with rat lung microsomes and a NADPH-generating system resulted in the *in vivo* covalent binding of [^{3}H]nilutamide metabolites to microsomal proteins; covalent binding required NADPH; it was decreased in the presence of $NADP^+$ (10 mM), or in the presence of the nucleophile glutathione (10 mM), but was unchanged in the presence of carbon monoxide. Under aerobic conditions, in contrast, the nitro anion free radical was reoxidized by oxygen, and its ESR signal was not detected. Covalent binding was essentially suppressed. Instead, there was consumption of NADPH and oxygen, and production of superoxide anion and hydrogen peroxide. We conclude that nilutamide is reduced by rat lung microsomes NADPH-cytochrome P450 reductase into a nitro anion free radical. In anaerobiosis, the radical is reduced further to covalent binding species. In the presence of oxygen, in contrast, this nitro anion free radical undergoes redox cycling, with the generation of reactive oxygen species.

Nilutamide is a non-steroidal anti-androgen derivative behaving as a competitive antagonist of the androgen receptor [1, 2]. This nitroaromatic compound is proposed in the treatment of metastatic prostatic carcinoma in association with castration [3, 4]. Therapeutic effects of nilutamide are overshadowed by the occurrence of several adverse drug reactions, including pulmonary interstitial fibrosis [5–7] and drug-induced hepatitis. Lung lesions occur in about 1.5% of recipients (Cassenne Laboratories, personal communication).

Toxicity of some nitroaromatic compounds such as nitrofuran and nitroimidazole derivatives depends on the reduction of the nitro group [8, 9]. The initial one-electron reduction of the nitro group forms a nitro anion free radical. Under anaerobic conditions, further reductions lead successively to the nitroso, the hydroxylamine and the amine metabolites. The nitroso and the hydroxylamine are reactive species which can covalently bind to glutathione and cellular macromolecules. Under aerobic conditions, however, molecular oxygen oxidizes the nitro anion free radical resulting in a redox cycle with regeneration of the nitroarene compound and formation of reactive oxygen species.

We have reported previously that rat liver microsomes catalyse the one-electron reduction of nilutamide to its corresponding nitro anion free radical [10]. It remains unknown, however, whether this radical is also formed in the lung. In the present study, we have investigated the metabolism of nilutamide by rat lung microsomes and have provided evidence that this anti-androgen is reduced to a nitro anion free radical capable of generating toxic reactive species both under aerobic and anaerobic conditions.

Materials and Methods

Materials. Male Sprague–Dawley Crl:CD (SD) BR rats, weighing 250–300 g were purchased from Charles River (Saint-Aubin-les-Elbeuf, France). Animals were fed *ad lib.*