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The Effects of 5-Fluorouracil and Doxorubicin on Expression of Human Immunodeficiency Virus Type 1 Long Terminal Repeat[1]

John Panozzo[2], Ender Akan[2], T. Daniel Griffiths[3], and Gayle E. Woloschak[2,4]

[2]Center for Mechanistic Biology and Biotechnology
Argonne National Laboratory
9700 South Cass Avenue
Argonne, IL 60437

and

[3]Department of Biological Sciences
Northern Illinois University
Dekalb, IL 60115

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[4]To whom correspondence should be addressed, at Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439-4833, Phone: 708-252-3312, Fax: 708-252-3387.

[5]The abbreviations used are HIV, human immunodeficiency virus type 1; LTR, long terminal repeat; CAT, chloramphenicol acetyl transferase; 5-FU, 5-Fluorouracil; DOX, doxorubicin; AIDS, acquired immune deficiency syndrome; PBS, phosphate-buffered saline.

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ABSTRACT— Previous work by many groups has documented induction of the HIV-LTR following exposure of cells to ultraviolet light and other DNA damaging agents. Our experiments set out to determine the relative activation or repression of the HIV-LTR in response to two classes of chemotherapeutic agents: Doxorubicin is a DNA-damage inducing agent, and 5-fluorouracil has an antimetabolic mode of action. Using HeLa cells stably transfected with a construct in which HIV-LTR drives expression of the chloramphenicol acetyl transferase reporter gene, we demonstrated an up to 10-fold induction following doxorubicin treatment at 24 h post-treatment. This induction was repressed by treatment with salicylic acid, suggesting a role for prostaglandin/cyclo-oxygenase pathways and/or NFKB in the inductive response. Induction by 5-fluorouracil, in contrast, was more modest (two-fold at most) though it was consistently elevated over controls.

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INTRODUCTION

Valerie *et al.* (1) stably transfected into HeLa cells a construct with the long terminal repeat (LTR) of the human immunodeficiency virus (HIV) type 1 driving transcription of the chloramphenicol acetyl transferase (CAT) reporter gene. Several groups have used this system to explore the ability of DNA damaging agents and other agents to induce HIV expression (2–6). The HIV promoter is activitated *in vivo* and *in vitro* in response to DNA damaging and tumor promoting agents (7–9), though the mechanism(s) by which this occurs is not known. In these experiments, we set out to determine whether the HIV-LTR is induced following exposure to DNA damaging agents, which act through different mechanisms.

Doxorubicin (DOX) or adriamycin, is a chemotherapeutic agent widely used for a broad variety of different neoplasias (10–12). In the past, cisplatin, DOX, bleomycin, and vincristine have been used as therapy for acquired immune deficiency syndrome (AIDS)-related Karposi sarcoma and other germ cell neoplasms (13). DOX has been shown to intercalate into DNA and can cause single-strand breaks, double-strand breaks, and sister chromatid exchange. This class of drug (anthracylines) can also interact with the cell membrane and participate in oxidation-reduction reactions (14).

5-Fluorouracil (5-FU) is similar to DOX in that it has been used clinically as a part of a chemotherapeutic regimen for many of the same AIDS-related

neoplasias as well as metastatic carcinomas of the breast and gastrointestinal tract, liver, prostate, pancreas, and oropharyngeal areas (14). In combination with 3´-azido 3´ deoxythimidine (AZT), 5-FU has been shown to greatly increase the antineoplastic activity in HIV-associated chronic myeloid leukemia (13). 5-FU, a pyrimidine analog, interferes with the synthesis of DNA by blocking the conversion of deoxyuridylic acid to thymidylic acid with the cellular enzyme thymidylate synthetase. It can also interfere with RNA synthesis, since the uracil analog has been shown to be present in mRNA that was transcribed and translated normally. The antineoplastic effect is believed to be due to thymine depletion, which halts DNA synthesis (15).

MATERIALS AND METHODS

Cell lines/culture conditions. All experiments were performed with human cervical carcinoma HeLa cells stably transfected with a construct containing geneticin resistance and the CAT reporter gene driven by the HIV-LTR promoter. The cell line was generously provided by Dr. K. Valerie (Virginia Commonwealth Medical Center) (1).

The cell cultures were maintained in Dulbecco's Modified Eagle's Medium containing 4500 mg/L D-glucose, 10% fetal bovine serum (GIBCO, Gaithersburg, MD) supplemented with sodium bicarbonate (0.73 mg/mL), penicillin (100 units/mL), streptomycin (100 units/mL), and geneticin (0.5 mg/ml). Cultures were grown in 100 x 20 mm Petri plates in 10 mL of

medium at 37 °C under 2.0% CO_2. Cell viability was determined by trypan blue dye exclusion.

CAT assay. Plates were harvested by washing with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), and the monolayer was trypsonized from the plates, washed, and counted on a hemocytometer as previously described (4–6). To control for the effects of cell killing by these drugs, only equal numbers of viable cells were used in the assay. Cells (1×10^6) were lysed in 0.25 M Tris-HCl by three freeze-thaw cycles. The cell lysate was incubated in a reaction containing 0.25 M Tris-HCl, 230 μM *n*-butyryl coenzyme A (Sigma), 40 μM ^{3}H-chloramphenicol (31 Ci/mmol; NEN Research Products) at 37 °C for 2 h. CAT was measured by the transfer of the butyryl moiety of the coenzyme to 3 H-chloramphenicol. The *n*-butyryl chloramphenicol partitions into xylene, while the unmodified chloramphenicol remains in the aqueous phase. The organic phase was added to 10 mL of Eco-lite scintillation fluid. The activity measured by liquid scintillation is directly related to the amount of CAT in the reaction mixture as shown by CAT controls provided in the kit (NEN Research Products). Results reported here are from a single experiment, but all CAT experiments were performed three times independently. We have found that direct cpm comparisons from one experiment to another are not possible. However, patterns or responses have been obtained at least three times.

DOX. The drug was obtained from Sigma Chemical Company. DOX stock solution at a concentration of 2 mg/mL was made in water, and the

appropriate dilutions were made by dilution in PBS and added directly to the culture media. We noted some batch-to-batch variation in the ability of DOX to induce HIV-LTR-CAT. This may be due to the fact that the drug is easily inactiviated by heat or light.

5-FU. The drug was obtained from Sigma Chemical Company. A stock solution at a concentration of 50 mg/mL was made, and the appropriate dilutions were made by dilution in PBS and added directly to the culture media.

Salicylic acid. Salicylic acid (2-hydroxy benzoic acid) was obtained from Sigma Chemical Company. It was dissolved in PBS and added to cultures at 2.5 mg/mL, a concentration shown previously by our group to inhibit cisplatin- and UV-mediated HIV-LTR induction (16).

Ultraviolet radiation. Exposures to 254 nm UV radiation were administered by using a General Electric (GE30T8) 30-W germicidal lamp that was contained in a sterile hood. The irradiations were at a distance of 55.6 cm from the source. The dose rate for all exposures to UVC radiation was 2.5 $Jm^{-2}sec^{-1}$. The media were removed from the 100-mm plates, and the plates were washed once with cold PBS and irradiated without covers. The media were replaced immediately after irradiation.

RESULTS

Both DOX and 5-FU caused increased activation of the HIV-LTR; however, the patterns and amounts of induction that they exhibited were different. Figure 1 demonstrates that DOX showed maximal expression of the CAT reporter gene at a concentration of 1 μg/mL at 24 h following addition of drug to culture medium. A 10-fold increase or decrease in concentration of the drug showed expression levels similar to that of controls. This optimum inducing concentration of the drug was then used to study the kinetics of the HIV-LTR induction. A 2-fold increase in expression was evident as early as 12 h (Fig. 2) following DOX addition. Within the first 24 h, maximal induction (9- to 11-fold) was observed 24 h following drug addition; submaximal expression (7-fold) was detected 18 h following drug exposure. At a concentration of 0.5 μg/mL, there was a 1.5-fold increase in expression compared with controls, and at 0.1 μg/mL there was no change compared with controls. The temporal expression shown in Figure 2 is consistent with results reported in the literature for the effects of other DNA damaging agents on HIV-LTR-CAT expression (1–7,14,16–18).

In the next series of experiments, we examined the effects of DOX combined with UV exposure (another HIV-LTR inducer) on the induction of CAT expression from the HIV-LTR. Figure 3 demonstrates 4- to 5-fold induction of CAT expression 48 h after exposure to the optimum concentration of DOX (1 μg/mL) as well as 6-fold induction 24 h following UV exposure. These are expected based on previous data in the literature (1–9) as well as

previous results (Fig. 1,2). However, it is interesting that DOX treatment had no additional effect on UV induction than UV exposure alone, suggesting that UV and DOX might act by similar mechanisms. Previous work from our group had shown that induction of HIV-LTR-CAT by either UV or cisplatin was inhibited by treating the cells with salicylic acid (16). In the next experiments, we set out to determine whether salicylic acid also inhibits DOX-mediated induction. Figure 4 demonstrates that salicylic acid (2.5 mg/mL) administered up to 6 h following DOX treatment (1 μg/mL) causes repression of the HIV-LTR induction. This time frame is consistent with studies that use UV and cisplatin (16).

In further experiments, we examined the effects of another chemotherapeutic drug, 5-FU, on HIV-LTR-CAT expression. Over a wide range of concentrations, only 10 μg/mL of 5-FU caused some induction (up to 2-fold) of the HIV-LTR 24 h after adding the drug to the culture medium (data not shown). While induction was modest, it was consistently found in each experiment. Previous work with methotrexate had demonstrated the need for longer incubations for induction of the promoter (17). In addition, a report in the literature (18) had demonstrated that 5-FU requires a longer time for induction of cellular apoptosis relative to cisplatin and DOX. Therefore, we designed experiments to examine the effects of longer drug exposures on HIV-LTR expression by using the peak dose of 10 μg/mL. Figure 5 demonstrates that a modest increase in expression was detected at 24, 48, and 72 h

following addition of drug. This induction was never greater than 2-fold, however.

Experiments similar to the UV/DOX experiments above (Fig. 3) were performed with 5-FU and UV. Figure 6 demonstrates modest induction with 10 μg/mL 5-FU at 24–72 h following treatment and 9-fold induction at 24 h following UV exposure. These results were expected based on the literature (1–9) and experiments depicted in Figures 4 and 5. Similar results were observed (Fig. 7) when a higher concentration of the drug (10 μg/mL) was used, and the experiments were left for 96 h. Salicylic acid inhibition experiments were not attempted with 5-FU due to the very modest levels of induction in the presence of 5-FU. These experiments suggest that 5-FU is not an effective inducer of HIV-LTR, even at high doses and with longer exposures.

DISCUSSION

The work done here demonstrates that exposure to DOX is able to induce activation of HIV-LTR expression, while 5-FU causes only a modest induction at best. This difference in response to the two drugs is consistent with what is known about the mechanisms of cytotoxicity in their use as anti-neoplastic agents. Our data have shown that DOX (an agent that causes DNA damage) induced significant and prolonged expression of the HIV-LTR, while

5-FU (an agent that is an antimetabolite) expression caused modest induction at best.

Previous work from our group has shown that most types of cell death correlate closely with the expression patterns of the HIV-LTR (4–6), a model that is consistent with the results presented in this manuscript. . DOX has the ability to act quickly in damaging DNA at any stage of the cell cycle, which ultimately leads to cell death, either necrotic or apoptotic. Induction of the HIV-LTR by DOX occurs during this early (24 h) time frame. 5-FU inherently cannot act quickly, especially in an asynchronous population of cells (18). 5-FU has the ability to act over a longer period of time by being incorporated into nucleic acids and stopping synthesis or interfering with transcriptional processes, which also ultimately leads to death. The low level of induction caused by 5-FU relative to DOX may be related either to its mechanism of action or to the time required for cell killing.

The combined effects of UV with either DOX or 5-FU on HIV-LTR transcription showed little effect of either drug in the UV response. Previous work from our group (16) has shown that cisplatin- and UV-mediated induction are inhibited by salicylate implicating either the prostaglandin/cyclo-oxygenase pathway or NFKB in the UV response. DOX is also inhibited with salicylate and thus may induce the HIV-LTR by this same pathway.

The direct applicability of this work to the clinical arena is questioned, since epithelial cells (like HeLa) are not known targets for HIV infection. We

are currently developing stably transfected CEM cells for future work. Nevertheless, these results suggest that possible contraindications exist when antineoplastic drugs are administered to HIV-positive individuals, and that *in vivo* studies comparing HIV expression of individuals receiving chemotherapy are needed to resolve the issue.

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FIGURE LEGENDS

Fig. 1. Effects of DOX on HIV-LTR expression. HeLa cells stably transfected with the HIV1-LTR-CAT construct were exposed to 10, 1, 0.1, 0.01 µg/mL of DOX for 24 h Control and experimental points were harvested at 24 h following addition of the drug to culture medium.

Fig. 2. Effects of DOX on HIV-LTR expression. HeLa cells stably transfected with the HIV1-LTR-CAT construct were exposed to 1.0 µg/mL for 4, 12, 18, and 24 h. Cells were exposed to 0.5 µg/mL for 24 h. Control and experimental points were harvested together upon completion of the DOX exposure.

Figure 3. Effects of DOX/UV exposure on HIV-LTR expression. HeLa cells stably transfected with the HIV1-LTR-CAT construct were exposed to DOX (1 µg/mL) and/or UV radiation (10 Jm^{-2}, 2.5 $Jm^{-2}sec^{-1}$) and harvested 48 h after the initiation of the culture. DOX was kept in culture for 48 h (e,g) or 24 h (d,t); UV exposure was done 24 h (b,g) or 48 h (c,f) prior to harvest. Control (0) cells were maintained in culture 48 h.

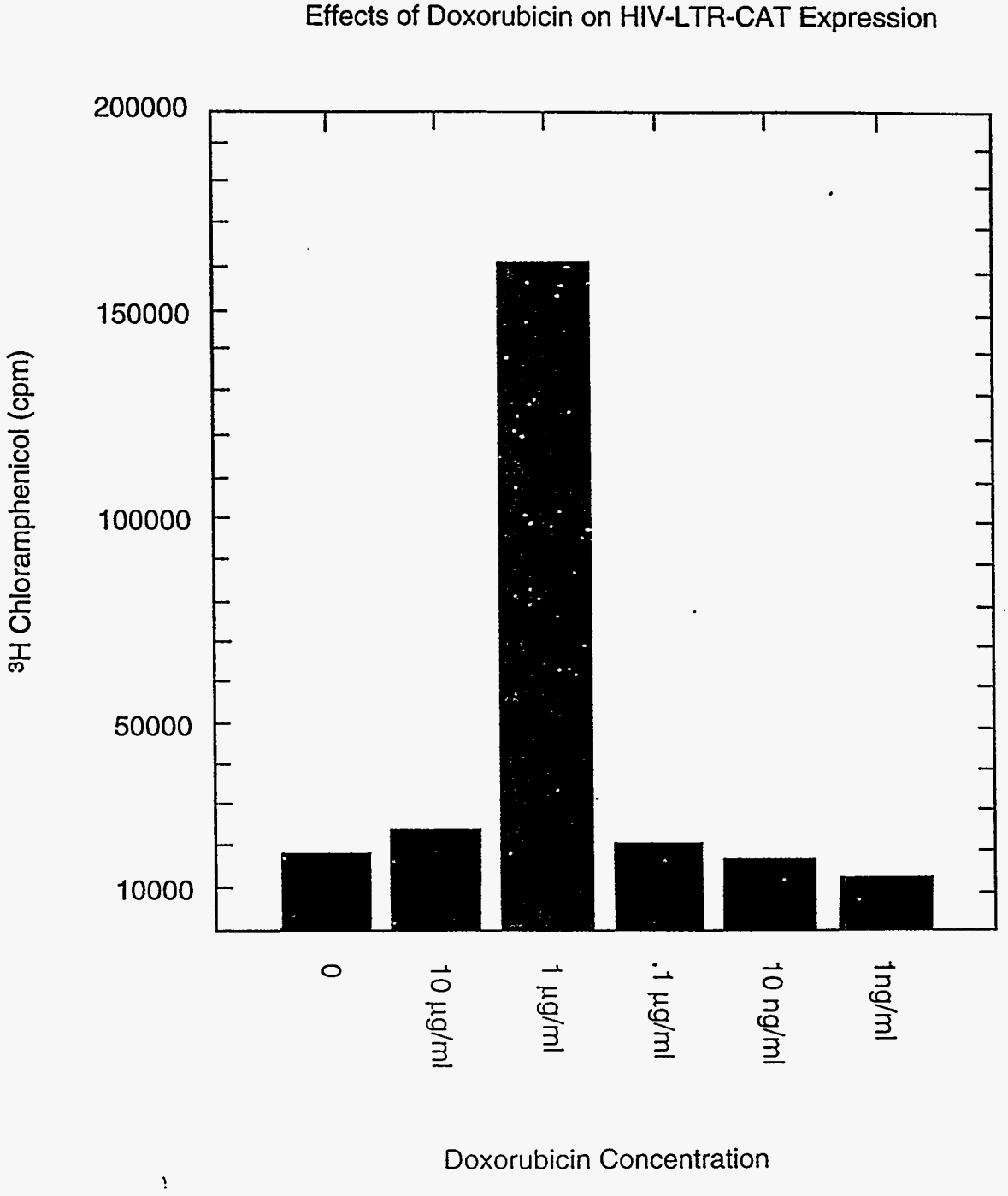
Fig. 4. Effects of salicylate on DOX response. Cells were untreated (0) or exposed to DOX (1 μg/mL) for 24 h (b). Salicylic acid (2.5 mg/mL) was administered at the same time as DOX (c), 6 h after DOX (d), or 12 h after DOX (e). All cells were harvested 24 h after addition of DOX.

Fig. 5. Effects of 5-FU on HIV-LTR expression. HeLa cells stably transfected with the HIV1-LTR-CAT construct were exposed to 10 μg/mL of 5-FU for 24, 48, and 72 h. Control points were harvested together upon completion of the 24 h (0–24 h) or 72 h (0–72 h) incubation.

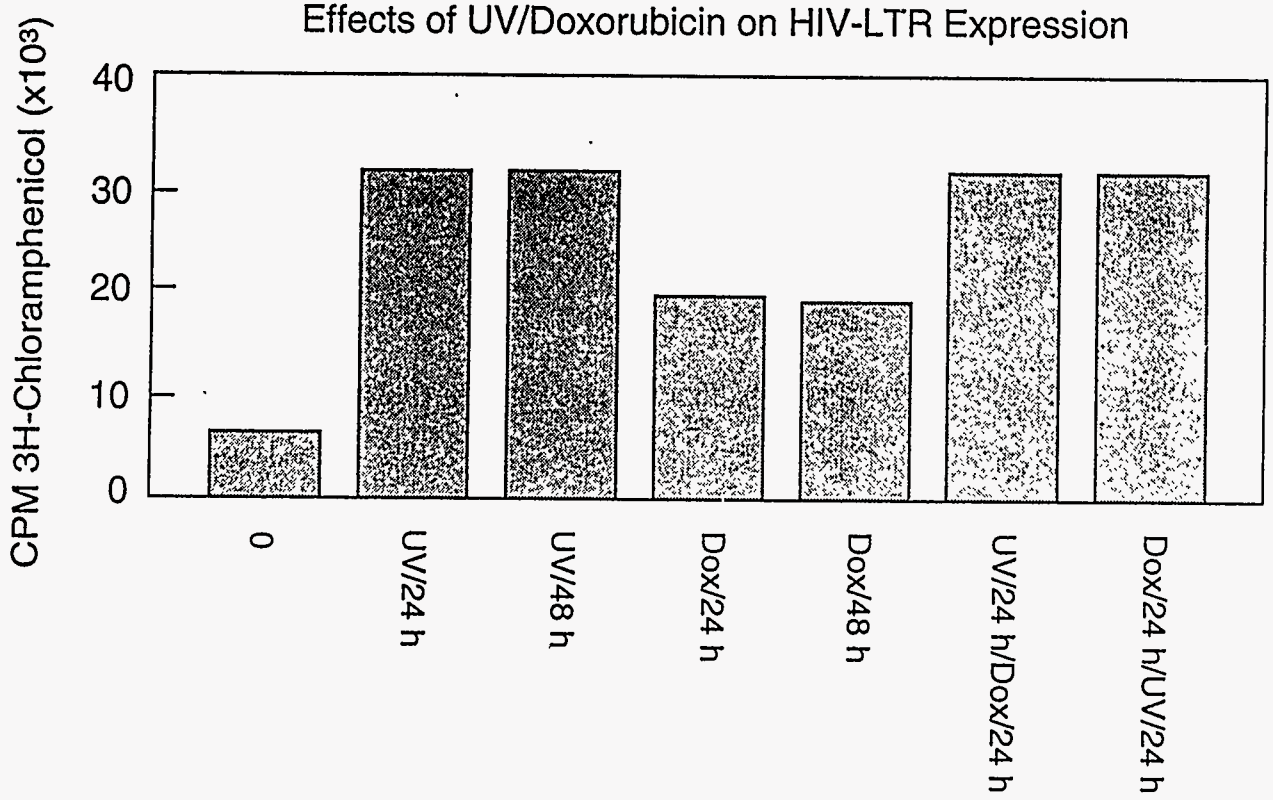
Fig. 6. Effects of 5-FU (10 μg/mL)/UV exposure on HIV-LTR-CAT expression. Cells were untreated (0), exposed to UV (10 Jm^{-2}, 2.5 $Jm^{-2}sec^{-1}$), or treated with 10 μg/mL 5-FU for 24 h or 48 h, with the UV dose given during the last 24 h culture period. Cells were harvested 24 or 48 h following 5-FU treatment and 24 h following UV exposure.

Fig. 7. Effects of 5-FU (1 μg/mL)/UV exposure on HIV-LTR-CAT expression. Conditions are identical to those in Fig. 6 except

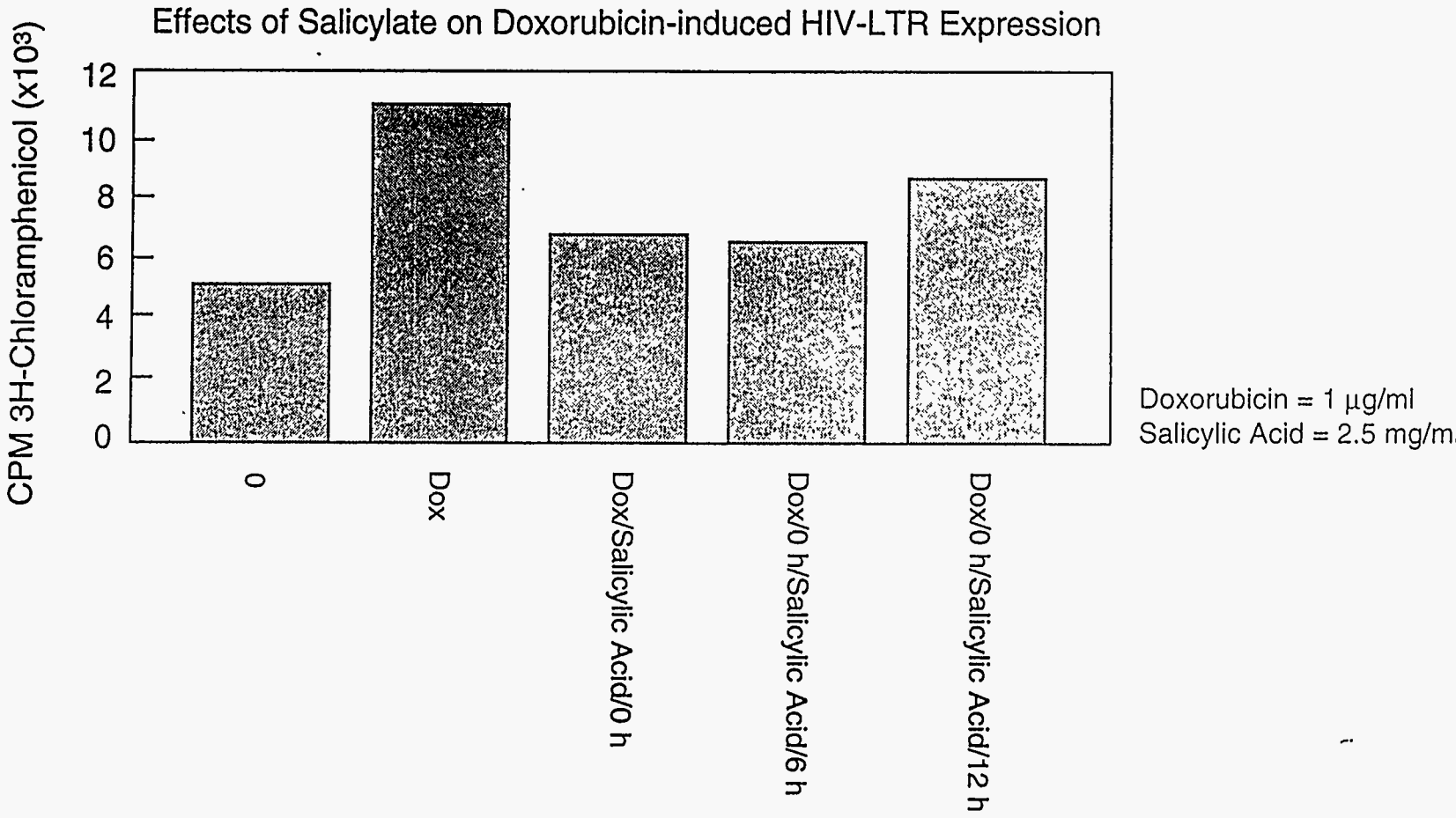
96 h culture was used (96 h with DOX, 24 h following UV exposure).



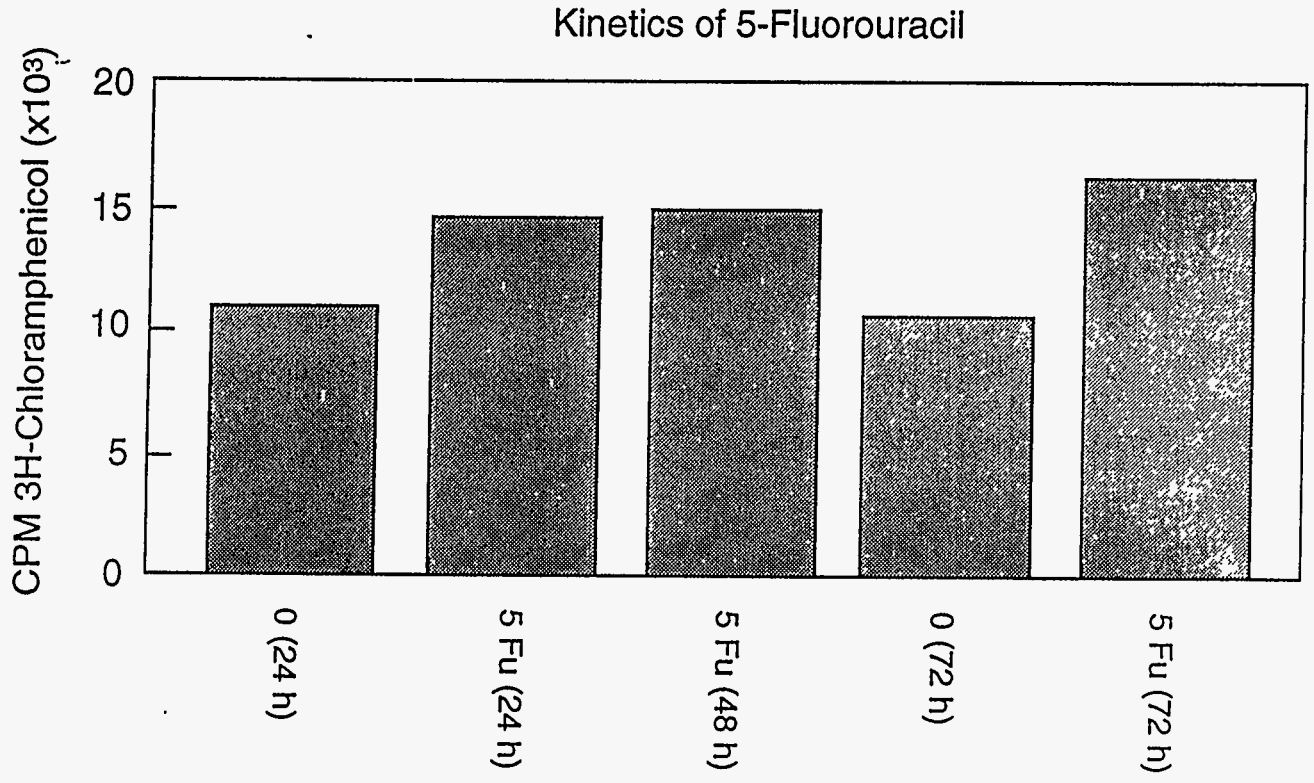
J. Panozzo et al. Fig. 1



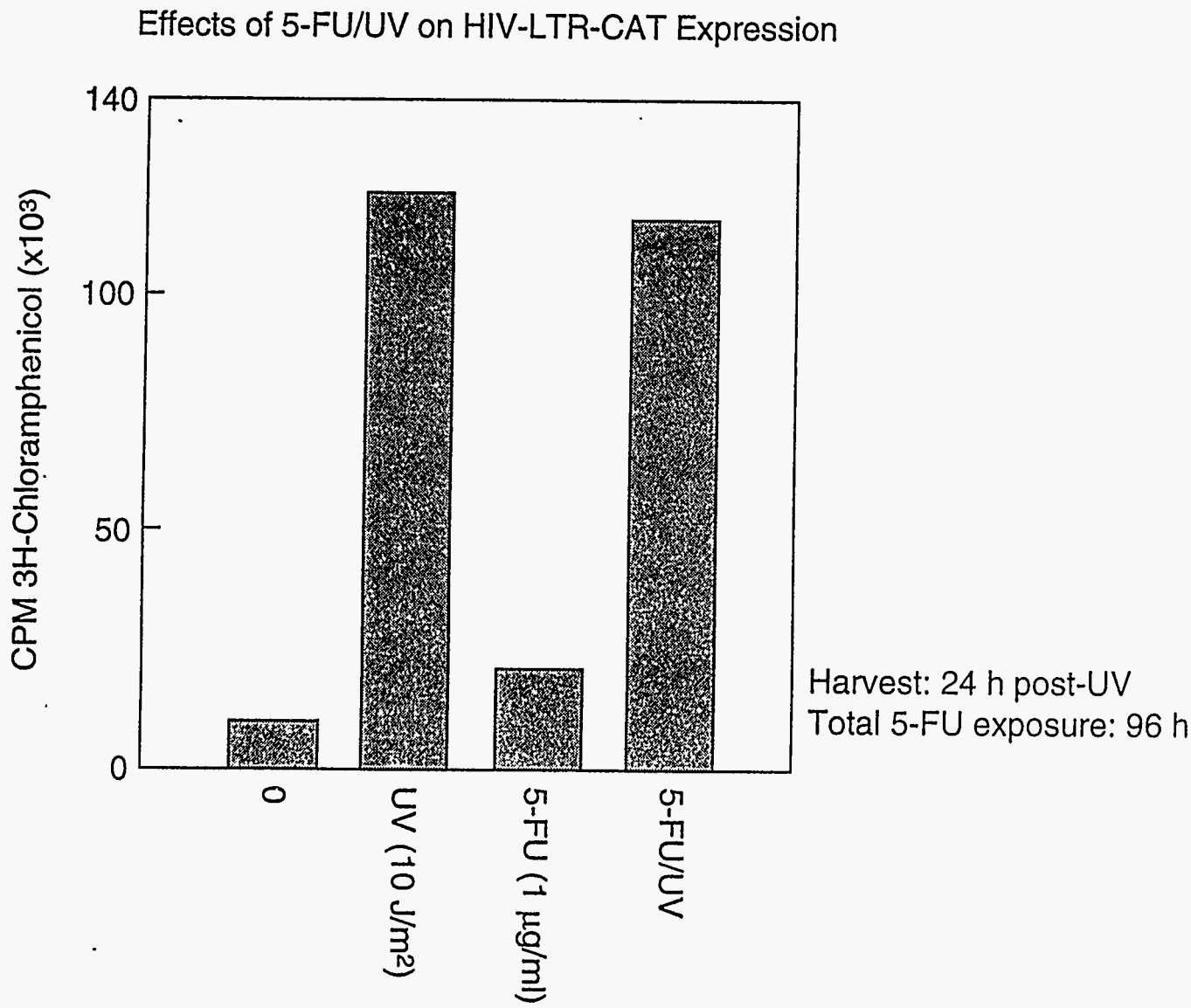
J. Panozzo et al. Fig. 3



J. Panozzo et al. Fig. 4



J. Panozzo et al. Fig. 5



J. Panozzo et al. Fig. 7