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Effect of Thymidine on Uptake, DNA Alkylation, and DNA Repair in L1210 Cells Treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea or 3′-[3-(2-Chloroethyl)-3-nitrosoureido]-3′-deoxythymidine[1]

E. Michael August[2] and William H. Prusoff

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

In order to define the mechanism for the enhancement by thymidine (dThd) of the antitumor activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine (3′-CTNU) in mice, we have investigated the effect(s) of dThd on the uptake of nitrosourea by L1210 cells in culture, DNA alkylation, and repair of the alkyl lesion. Using a rapid centrifugation technique through silicone:paraffin oil, we observe a 1.3- and 1.5-fold increase in the uptake of radioactivity from 0.1 mM [*chloroethyl*-^{14}C]BCNU in the presence of a 5- and 25-fold excess of dThd, respectively. Similarly, an enhancement of DNA alkylation was observed upon treatment of L1210 cells for up to 3 h with 0.1 mM [*chloroethyl*-^{14}C]BCNU from 70 pmol ^{14}C/mg DNA in control to 85, 95, and 120 pmol ^{14}C/mg DNA with equimolar 5- and 25-fold excess dThd, respectively. No effect of dThd on the uptake of 0.1 mM [*chloroethyl*-^{14}C]-3′-CTNU was observed, although a small increase in DNA alkylation at 3 h was evident. DNA repair, as measured by the amount of radioactivity remaining associated with the DNA after an initial 2-h treatment with labeled BCNU was largely unaffected by dThd. Although dThd appears to enhance the cellular uptake of BCNU and the alkylation of DNA by both BCNU and 3′-CTNU, dealkylative repair proceeds unhindered in the presence of dThd.

INTRODUCTION

The clinical efficacy of certain (2-haloethyl)nitrosourea derivatives is well established. These include, for example, BCNU[3] (1), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and 1-(2-chloroethyl)-3-[4′-*trans*-(methylcyclohexyl)]-1-nitrosourea in the treatment of Hodgkin's disease (2, 3), brain tumors (4), and lymphomas (7). The history of the origin and development of the nitrosoureas as well as reviews of their structure-activity relationships, mechanism(s) of action, experimental antitumor activity, pharmacology, and clinical usage have been reported (5–8).

The ability of dThd to modulate the activity of various cytotoxic drugs in a synergistic fashion has also been reviewed (9). The role of dThd as a biochemical modulator of standard chemotherapeutic agents such as 5-fluorouracil (10), 1-β-D-arabinofuranosylcytosine (11), and methotrexate (12) have been clinically exploited. Thymidine itself, on the basis of activity against human tumor xenografts in mice (13), was introduced into clinical trial by the National Cancer Institute in 1978.

Lin *et al.* (14) reported that the coadministration of dThd with 3′-CTNU, a chloroethylnitrosourea analogue of dThd, did not prevent the initial weight loss caused by 3′-CTNU alone but did prevent the lethality otherwise produced in non-tumor-bearing mice. A single dose of dThd (2 g/kg) administered to P-388- or L1210-bearing mice had no antitumor activity, but when coadministered with 3′-CTNU an enhancement of the antitumor activity was observed. Lin and Prusoff (15) further reported that the coadministration of dThd (2 g/kg) with BCNU (10, 15, and 20 mg/kg) to mice bearing the L1210 leukemia or the B16/F10 melanoma resulted in a significant increase in the length of survival of the mice, as well as a marked increase in the number of >60-day survivors. These studies show that the effect of dThd on the anticancer activity of the nitrosoureas is not limited to nucleoside nitrosourea derivatives alone.

The present studies seek to define the biochemical basis for the enhancement of the antitumor activity of BCNU and 3′-CTNU by dThd. Two hypotheses may be suggested to account for this enhancement: (*a*) that dThd augments the reactivity of the nitrosourea by either facilitating attack at known target sites or making available new or additional sites within the cell; or (*b*) that dThd decreases the ability of the neoplastic cell to repair the DNA damage produced by the nitrosourea. Because the alkylation of DNA is considered the primary mode of action of the nitrosoureas (3, 6), the effect of dThd on DNA alkylation, repair of the alkylated lesion, as well as the effect of dThd on the uptake of BCNU and 3′-CTNU by L1210 cells in culture forms the basis for these studies. A preliminary account of this research has been presented (16).

MATERIALS AND METHODS

Chemicals. [*chloroethyl*-^{14}C]BCNU (17 Ci/mol) was obtained, through the courtesy of Dr. R. Cysyk, from the Laboratory of Medicinal Chemistry and Pharmacology, Drug Metabolism Section, National Cancer Institute. Nonradioactive BCNU was obtained from Bristol Laboratories, Syracuse, NY. [*chloroethyl*-^{14}C]-3′-CTNU (2.8 and 12.3 mCi/mmol) was synthesized by procedures developed in this laboratory (17). Thymidine was obtained from Sigma Chemical Co. All other chemicals were of reagent grade unless otherwise specified. Phenol was redistilled and stored at −70°C prior to use.

Cells. Murine leukemia L1210 cells were grown in Fischer's medium supplemented with 10% horse serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 atmosphere as suspension cultures. Asynchronous exponentially growing cells were used in all experiments, with a doubling time of approximately 13 h. Cell counts were made with a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

Drug Uptake Studies. Drug uptake was determined by a modification of the procedure of Darnowski *et al.* (18). "Oil stop" tubes were prepared by layering 100 μl of silicone:paraffin oil (Dow Corning DC550 silicone oil:Fischer light paraffin oil, 78:22) over 50 μl of 7% $HClO_4$ in a 400-μl microcentrifuge tube. L1210 cells were suspended at 5×10^6 cells/ml in Fischer's media with 10% horse serum, to which was added radiolabeled drug ± dThd at the appropriate concentration to initiate the time course. Incubations were carried out at 37°C in a shaking water bath. At timed intervals, 200 μl of incubating mixture containing 1×10^6 cells were gently layered over the "oil stop" mixture and the cells were separated from the incubation media by centrifugation at 15,000 × *g* for 15 s in an Eppendorf model 5412 microcentrifuge. The tubes were rapidly frozen in dry ice and cut through the oil layer, and each portion was placed in a plastic miniscintillation vial containing 0.2 ml of H_2O and vortexed vigorously. The radioactivity in each portion was determined in 5 ml of Optifluor (Packard Instrument Co.,

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[2] To whom requests for reprints should be addressed, at the Department of Pharmacology, Yale University School of Medicine, Sterling Hall of Medicine, P. O. Box 3333, New Haven, CT 06510.

[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; 3′-CTNU, 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine; dThd, thymidine.

Downers Grove, IL) in a Beckman model LS7500 liquid scintillation counter.

In separate determinations, the total and extracellular water spaces of the cell pellet were measured by incubating cells with ^3H_2O (5.5×10^3 dpm/μl) and [^{14}C]inulin (1.1×10^3 dpm/μl) for approximately 5 min, centrifuging as described above, and determining dpm for each isotope in the cell pellet. The intracellular volume of the pellet (μl cell H_2O) was defined as [(^{3}H-related) − (^{14}C-related)dpm] and volume of trapped media or extracellular H_2O space as (^{14}C-related) dpm (18). These values were found to be 0.72 ± 0.04 and 0.36 ± 0.04 μl (N = 16), respectively. Drug uptake data were corrected for extracellular radioactivity present in the pellet. Cell viability, as measured by trypan blue exclusion, was 95% or better using either BCNU or 3′-CTNU ± dThd over the times indicated.

DNA Alkylation Studies. L1210 cells (4×10^7/ml) in Fischer's medium with 10% horse serum were incubated with radiolabeled drug ± dThd for 6 h at 37°C. At 0, 1, 3, and 6 h, 1×10^8 cells were removed, washed twice with ice-cold phosphate-buffered saline, and immediately frozen at −70°C prior to extraction. DNA was extracted by the phenol technique of Blin and Stafford (19). A portion of the purified DNA was hydrolyzed in 0.5 M $HClO_4$ for 70 min at 68°C. The amount of DNA present was determined by the Burton diphenylamine procedure (20) and the amount of radioactivity associated with the DNA was determined in Optifluor (Packard Instrument Co.)

DNA Repair Studies. L1210 cells (4×10^7/ml) in Fischer's media supplemented with 10% horse serum were incubated with radiolabeled nitrosourea ± dThd for 2 h, after which the cells were washed once with ice-cold phosphate-buffered saline, resuspended at 1×10^7 cells/ml in drug-free media containing the appropriate concentration of dThd, and postincubated at 37°C for various periods of time to allow repair. At the indicated times over a 24-h period, 1×10^8 cells were removed and the DNA was extracted and quantitated as above. The extent of DNA repair was expressed as the amount of radioactivity remaining associated with the DNA as described previously (21).

RESULTS

Effect of dThd on Nitrosourea Uptake by L1210 Cells. The uptake of radioactivity by L1210 cells upon incubation with 0.1 mM [*chloroethyl*-^{14}C]BCNU in the presence of 0, 0.1, 0.5, and 2.5 mM dThd is shown in Fig. 1. dThd causes a concentration-dependent increase in the amount of radioactivity associated with the cell pellet. The cell-associated radioactivity increased by 90 min from 1980 pmol ^{14}C/μl cell water when cells were incubated in the absence or presence of equimolar dThd to 2550 and 2730 pmol ^{14}C/μl cell water in the presence of a 5- and 25-fold excess of dThd, respectively. This represents a 1.3- and 1.5-fold increase in uptake. Begleiter *et al.* (22) have shown BCNU to be taken up into L5178Y cells via passive diffusion; thus our observed concentration of radioactivity in the L1210 cells to 20 times the extracellular concentration is presumably due to reaction of the nitrosourea with membrane and/or intracellular components.

In similar experiments with [*chloroethyl*-^{14}C]-3′-CTNU, no such enhancement of uptake by dThd was observed, even in the presence of a 25-fold excess of dThd (Fig. 2). Uptakes of radioactivity from 3′-CTNU in the presence of equimolar and a 5-fold excess of dThd were equivalent to control as well (data not shown).

Effect of dThd on the Alkylation of L1210 DNA. The effect of dThd on the extent of DNA alkylation upon exposure of L1210 cells to 0.1 mM [*chloroethyl*-^{14}C]BCNU over a 6-h period is shown in Fig. 3. At the times indicated, DNA was extracted from approximately 10^8 cells and quantitated as described in "Materials and Methods." The amount of radioactivity associated with the DNA was found to be influenced by dThd in a manner similar to that for uptake. The level of alkylation was found to increase with increasing dThd at 3 h of incubation from 70 pmol ^{14}C/mg DNA in the absence of dThd, to 85, 95, and 120 pmol ^{14}C/mg DNA in the presence of 0.1, 0.5, and 2.5 mM dThd, respectively. This represents a 1.4- and 1.7-fold enhancement of DNA alkylation in the presence of a 5- and 25-fold excess of dThd and is consistent with the enhancement of uptake discussed above.

After 3 h of incubation the levels of alkylation decline, so that by 6 h the extent of radioactivity associated with the DNA is equivalent to control at all dThd concentrations examined.

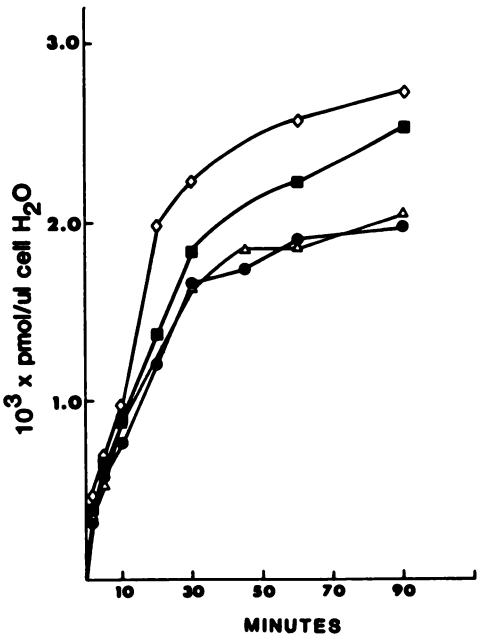

Fig. 1. Uptake of radioactivity from 0.1 mM [*chloroethyl*-^{14}C]BCNU by L1210 cells in the presence of varying concentrations of dThd. ●, no dThd; △, 0.1 mM dThd; ■, 0.5 mM dThd; ◇, 2.5 mM dThd. Uptake was determined as described in "Materials and Methods." Cell water volume was calculated by measuring uptake of ^3H_2O, corrected for extracellular volume by measuring [^{14}C]inulin content of pellet. *Data points* reflect the average of 3–4 determinations with SD = ±40–60 pmol/μl cell water.

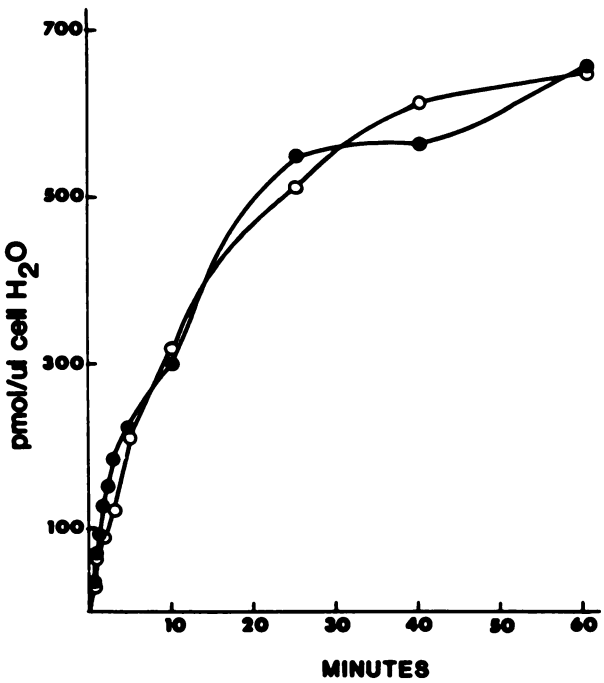

Fig. 2. Uptake of radioactivity from 0.1 mM [*chloroethyl*-^{14}C]-3′-CTNU (2.8 mCi/mmol) by L1210 cells in culture alone (●) and in the presence of 2.5 mM dThd (○). Procedures as described in "Materials and Methods." *Data points* reflect the average of 3–4 determinations.

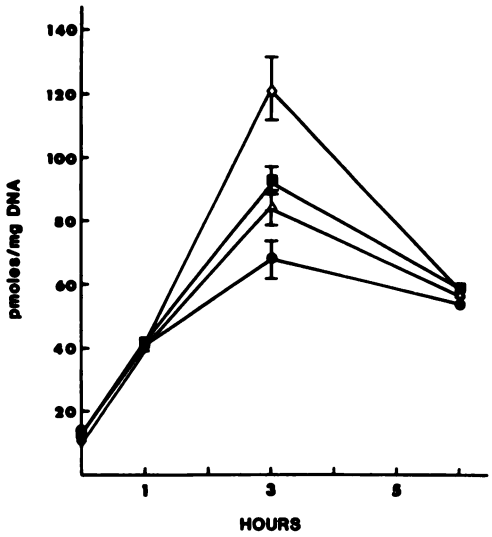

Fig. 3. Effect of dThd on the alkylation of L1210 DNA by 0.1 mM [*chloroethyl*-^{14}C]BCNU. Exponentially growing cells at 4×10^7 cells/ml were exposed to 0.1 mM [^{14}C]BCNU (specific activity, 17 Ci/mol) for 6 h. At the indicated times cells were removed from the incubation, the DNA was isolated, and the radioactivity associated with the DNA was determined as described in "Materials and Methods." DNA recovery was in the range of 0.8–1.0 mg DNA/10^8 cells for all samples. *Data points* reflect the average of 3–4 determinations with *error bars* ± SD shown for 3-h time points only for clarity. ●, no dThd; △, 0.1 mM dThd; ■, 0.5 mM dThd; ◇, 2.5 mM dThd. Cells were >80% viable over the 6 h of examination.

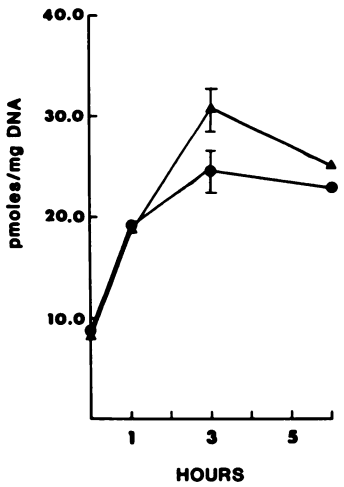

Fig. 4. Effect of dThd on the alkylation of L1210 DNA by 0.1 mM [*chloroethyl*-^{14}C]-3′-CTNU (12.3 mCi/mmol). Procedures were as described in Fig. 3. ●, no dThd; ▲, 2.5 mM dThd. *Data points* reflect the average of 2 determinations with *error bars* ± SD. Cells were >80% viable over the course of the study.

This probably reflects DNA repair and suggests either that dThd may not affect the dealkylative repair processes or that the cell has the ability to overcome an initial inhibition of repair activity.

A dThd-mediated increase in DNA alkylation was also observed in similar experiments with [*chloroethyl*-^{14}C]-3′-CTNU (Fig. 4). At 3 h of incubation, thymidine (2.5 mM) increased the level of alkylation from 24 pmol ^{14}C/mg DNA to 31 pmol ^{14}C/mg DNA. As in the BCNU experiments, the level of alkylation decreased by 6 h to approximately 23 and 25 pmol ^{14}C/mg DNA, once again indicating repair.

Effect of dThd on the Repair of Alkylated DNA. To assess DNA repair, L1210 cells were incubated with 0.1 mM ^{14}C-labeled BCNU and various concentrations of dThd for 2 h, washed free of the drug, resuspended in media containing dThd (0.0, 0.1, 0.5, or 2.5, mM), and postincubated to allow repair. At the times indicated in Fig. 5, DNA was isolated from 10^8 cells as before and the amount of radioactivity remaining associated with the DNA was determined. Thus, the data in Fig. 5 represent the repair of the radioactive DNA lesions following removal of the BCNU.

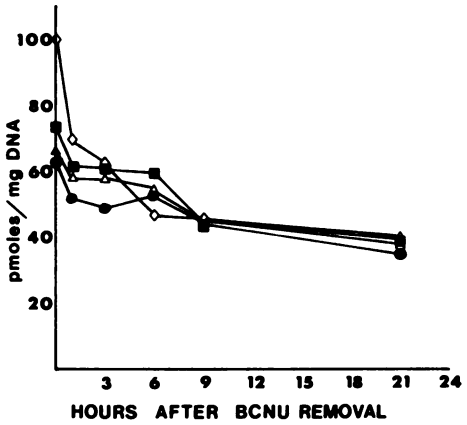

Fig. 5. Effect of dThd on the repair of alkylated DNA derived from L1210 cells incubated with 0.1 mM [*chloroethyl*-^{14}C]BCNU. L1210 cells were treated as described in "Materials and Methods," and at 0, 1, 3, 6, 9, and 21 h a sample containing 1×10^8 cells was removed, the DNA was extracted, and the radioactivity remaining associated with the DNA was determined. *Data points* reflect the average of triplicate determinations, SD = ±8 pmol ^{14}C/mg DNA. ●, no dThd; △, 0.1 mM dThd; ■ 0.5 mM dThd; ◇, 2.5 mM dThd. Cells were >80% viable over the course of the study.

In general, the cells removed >50% of the alkyl lesions during the 24-h postincubation period, with an initial burst of repair activity within the first 3 h followed by a more gradual repair period. At 0 h postincubation, the levels of DNA alkylation by [*chloroethyl*-^{14}C]BCNU (0.1 mM) were 63, 67, 73, and 101 pmol ^{14}C/mg DNA for cells incubated with 0.0, 0.1, 0.5, and 2.5 mM dThd, respectively. Equimolar and 5-fold excess dThd had no apparent effect on the rate or extent of repair. Thymidine, at a 25-fold excess, appeared to enhance the extent of repair during the first hour after BCNU removal, although the activity of O^6-alkyl DNA alkyltransferase, the enzyme responsible for removal of the major alkyl lesions induced by chloroethylnitrosoureas, has been shown to be more active on more highly alkylated substrates (23). The alkylation damage was repaired back to the same absolute level after 9 h under all conditions examined.

DISCUSSION

The ability of thymidine to modulate the activity of a wide variety of cytotoxic agents in a synergistic fashion has been reviewed recently (9). Lin and Prusoff (15) have shown that the coadministration of dThd with BCNU to mice bearing the L1210 or B16/F10 neoplasm markedly increased the anticancer activity of this nitrosourea. Lin *et al.* (14) previously demonstrated a similar augmentation of activity when dThd was coadministered to tumor-bearing mice with 3′-CTNU, a chloroethylnitrosourea analogue of dThd.

The uptake of BCNU into tumor cells has been shown to be a non-carrier-mediated, diffusion-controlled process (22), whereas the uptake of dThd is mediated by a nucleoside-specific transporter which accepts a wide range of nucleoside analogues (24). On structural grounds, 3′-CTNU might be expected to be a substrate for the nucleoside transport system, although recent experiments in our laboratory indicate that 3′-CTNU enters cells via passive diffusion also.[4] Our finding that dThd can augment the uptake of BCNU-related radioactivity is somewhat surprising, since these compounds are presumed to enter the cell via different pathways. Although the mechanism responsi-

[4] E. M. August, unpublished observations.

ble for this observation is unknown, several possibilities may be considered: (*a*) whether dThd causes alterations at the membrane leading to enhanced diffusion: (*b*) an involvement of the secondary nucleoside transport activity reported by Belt (25) in L1210 and several other cultured neoplastic cell lines, or (*c*) whether dThd enhances the uptake of intact BCNU or one of its (radioactive) decomposition products by affecting the breakdown of BCNU in the media.

Figs. 3 and 4 show that dThd enhances the alkylation of DNA by BCNU and, to a lesser extent, by 3′-CTNU. This enhancement is consistent with increased uptake of intact nitrosourea and also with the initial finding of increased antitumor activity. That 3′-CTNU uptake is not enhanced by dThd under the conditions examined (but DNA alkylation is) suggests that among the many potential effects which dThd may produce, one may consider an alteration of the subcellular distribution of the nitrosourea. Studies are currently in progress to determine the chemical nature of the species taken up by the cells, *i.e.*, intact drug, the alkylating or carbamoylating species, or other products resulting from nitrosourea breakdown, as well as the effect of dThd on the subcellular distribution of the nitrosoureas and their reaction products.

The enhancement of BCNU antitumor activity by dThd is not due simply to an increase in alkylation, however. Fig. 5 shows that even though dThd increases the alkylation of cellular DNA, the ability of the cell to repair the alkylated lesion is unhindered by dThd. The data in Fig. 5 reflect gross removal of ^{14}C label from alkylated DNA. As noted previously by Ahlgren *et al.* (21), such data merely define an upper limit to the extent of DNA repair activity, giving no insight into the physical state of the DNA during or after the repair process. There may be persistent strand breaks or cross-linking which go undetected by this method. The effects of dThd on nitrosourea-induced DNA strand breaks/cross-links and their repair are currently under study in this laboratory. However, since initial removal of the major alkyl lesion precludes conversion to a lethal cross-link (26), this repair activity may be of primary importance for repair of damaged DNA following exposure to haloethylnitrosoureas.

The chloroethylnitrosoureas comprise one of the most potent and effective classes of antitumor agents (2, 4). However, toxicity, including bone marrow suppression, severely limits their clinical usefulness (1–6). The antitumor activity of nitrosoureas has been potentiated by various agents including caffeine (27), 6-aminonicotinamide (28), and now dThd (14, 15). On the basis of its low systemic toxicity in humans and the ability to attain relatively high serum levels (9), dThd shows great promise for clinical use in the potentiation of BCNU activity. Elucidation of the molecular basis for this effect may afford the opportunity to potentiate the activity of the nitrosoureas as well as other cytotoxic agents and thus achieve therapeutic effect at a lower dose. Such an approach may circumvent the problems of toxicity which now limit the clinical usefulness of these drugs.

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