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SHORT COMMUNICATION

Chemosensitization of Cancer Cells by the Staurosporine Derivative CGP 41251 in Association with Decreased P-Glycoprotein Phosphorylation

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ABSTRACT. The multidrug resistance (MDR) phenotype of cancer cells often correlates with the level and activity of protein kinase C (PKC). We studied the ability of the staurosporine derivative PKC inhibitor CGP 41251 to reverse the MDR phenotype in MCF-7 human breast carcinoma and CT-26 murine colon adenocarcinoma cells and their doxorubicin (DXR)-selected MDR variants. Nontoxic concentrations of CGP 41251 significantly enhanced the cytotoxic properties of DXR, actinomycin D, vinblastine, and vincristine but not those of 5-fluorouracil. CGP 41251 increased intracellular concentrations of [^{14}C]DXR but did not cause significant differences in P-glycoprotein (P-gp) expression. Pretreatment of MCF-7^{adr} cells with phorbol 12-myristate 13-acetate reduced the CGP 41251-mediated intracellular accumulation of [^{14}C]DXR. At concentrations that induced drug uptake, CGP 41251 significantly decreased the level of P-gp phosphorylation in the cells but did not compete with [^{3}H]azidopine for photoaffinity labeling of P-gp. These data provide evidence that CGP 41251 reverses the MDR phenotype by modulating the phosphorylation of P-gp and/or other PKC substrates critical to the maintenance of the MDR phenotype. *Copyright @ 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:245–247, 1997.

KEY WORDS. multidrug resistance; PKC; staurosporine derivative; MDR-reversal; drug accumulation

The MDR† phenotype of tumor cells is often associated with the overexpression of a 170-kDa glycoprotein, P-gp, which is encoded by the *mdr-1* gene [1–3]. The glycoprotein is an energy-dependent pharmacokinetic efflux pump that spans the plasma membrane and extrudes a broad range of structurally unrelated intracellular chemotherapeutic drugs and other compounds to the extracellular space, thus reducing their intracellular accumulation [2, 3]. Many compounds designed to reverse MDR, such as calcium channel blockers and cyclosporins, have produced nonspecific toxicities because of their ability to directly bind P-gp [3]. Interfering with the regulatory post-translational modifications of P-gp provides a different, potentially less toxic, approach. For example, many MDR cells express high levels of PKC and PKC activity [4–7], suggesting a role for PKC in regulating the expression or activity of P-gp [7–9]. PKC can phosphorylate P-gp on serine/threonine residues, and this phosphorylation correlates positively with P-gp activity [9, 10]. A candidate for modulating P-gp kinetics is the PKC inhibitor CGP 41251, a staurosporine derivative. Although its potency for PKC inhibition is only one-eighth that of staurosporine, its maximum tolerated dose is 250 times higher than that of staurosporine [11]. CGP 41251 has been shown to selectively inhibit PKC-α and -β activity, to mediate antiproliferative effects against cancer cells *in vitro* and *in vivo* [11, 12], and to reverse the MDR phenotype in the CCRF-VCR1000 lymphoblastoid cell line [13]. Because the mechanism by which CGP 41251 reverses resistance to MDR-related drugs is unknown, we conducted a series of experiments in human and murine cancer cells addressing this question.

First, we determined the relative antiproliferative effects of CGP 41251 (supplied by Ciba-Geigy, Inc., Basel, Switzerland) against parental and MDR cells. The human MCF-7 and MCF-7^{adr} cells (the gift of Dr. Kenneth H. Cowan, National Cancer Institute, Bethesda, MD) were found to be more resistant to CGP 41251 than the murine parental CT-26 colon carcinoma cells [14] and their MDR variant CT-26 R500 cells [15] but no discernible differences in the dose response to CGP 41251 were found between parental and MDR cells. In preliminary experiments, we found that 30 and 125 nM concentrations of CGP 41251 were not toxic against CT-26 and MCF-7 cells, respectively. We treated the cells with these nontoxic concentrations of CGP 41251 and found that it sensitized MDR

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† *Abbreviations:* DXR, doxorubicin; 5-FU, 5-fluorouracil; MDR, multidrug resistance; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); P-gp, P-glycoprotein; PKC, protein kinase C; and TPA, phorbol 12-myristate 13-acetate.

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cells up to 15-fold against DXR, vinblastine, vincristine, and actinomycin D. No increased sensitivity was observed with 5-FU (Table 1). Next, we incubated control and CGP 41251-treated cells with medium containing [^{14}C]DXR. Nontoxic concentrations of CGP 41251 significantly increased the intracellular accumulation of [^{14}C]DXR in the MDR cell lines but not in the parental cell lines, directly correlating with the increased sensitivity of the cells (Table 1). The human MCF-7^{adr} cells have been shown to express 10-fold higher levels of PKC-α than the parental MCF-7 cells [9]. The relationship of PKC activity with intracellular drug accumulation was supported by our experiments showing that pretreatment with TPA (resulting in stimulation of PKC activity) reduced by 50% the ability of CGP 41251 to increase intracellular accumulation of [^{14}C]DXR (data not shown).

Staurosporine has been shown to increase intracellular drug accumulation by competing with chemotherapeutic agents for binding to P-gp as demonstrated by inhibition of [^{3}H]azidopine [8] or [^{3}H]vinblastine [16] binding. MDR reversal agents that directly compete for P-gp drug binding sites have been associated with severe toxicity *in vivo*, precluding their use in the treatment of clinical drug resistance [3]. To determine whether CGP 41251 could be distinguished from staurosporine [8], verapamil, and other P-gp-binding drugs [3] in its mechanism of MDR reversal, we monitored photoaffinity labeling of P-gp from membranes of MCF-7^{adr} cells by [^{3}H]azidopine in the presence of CGP 41251. As shown in Fig. 1, CGP 41251 at the concentration used to reverse MDR (125 nM) did not reduce the binding of [^{3}H]azidopine to P-gp under conditions where vinblastine, a known P-gp substrate, did. At 3-fold the concentration used to reverse MDR (>350 nM), CGP 41251 did compete with [^{3}H]azidopine. These results provide evidence that CGP 41251 can reverse MDR by a mechanism that does not involve binding interactions with P-gp. These

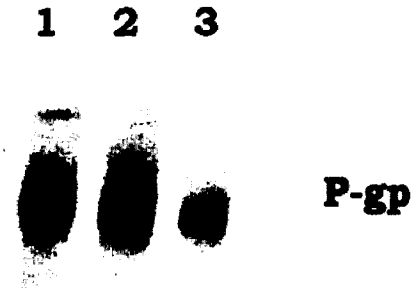

FIG. 1. Photoaffinity labeling of MCF-7^{adr} cells by [^{3}H]azidopine. MCF-7^{adr} membrane preparations (100 μg) were incubated for 30 min at 23° prior to UV irradiation at 366 nm with 25 μCi [^{3}H]azidopine in the absence (lane 1) or presence (lane 2) of 125 nM CGP 41251. Vinblastine (3 μg/mL) was used as a positive control (lane 3). The proteins were immunoprecipitated by C219 anti-P-gp antibody, separated on SDS–PAGE gel, fixed, stained, and exposed for 7 days at −70° using Hyperfilm-MP.

results do not entirely agree with a previous report that at a lower concentration, CGP 41251 did compete with vinblastine binding [16].

Treatment with some MDR-reversing agents, including staurosporine, has been shown to influence the expression of P-gp [17, 18]. We examined whether CGP 41251 reversed MDR by modulating P-gp levels. Northern blot and western blot analyses demonstrated that treatment of cells with CGP 41251 did not reduce the level of MDR-1 mRNA and protein, respectively (data not shown), in agreement with a previous report [13].

PKC-α and PKC-β have been shown to modulate drug resistance [5–7] and to be selectively inhibited by CGP 41251 [13]. The PKC-α isozyme has also been shown to be highly overexpressed and to be pivotal in the phosphorylation of P-gp in MCF-7^{adr} cells [9]. We therefore examined the ability of CGP 41251 to inhibit P-gp phosphorylation in MCF-7^{adr} cells [8]. The results in Fig. 2 show a 45%

TABLE 1. Enhancement of *in vitro* chemosensitivity and intracellular drug accumulation in tumor cells treated with CGP 41251

	Enhancement ratio*†						
	IC_{50}‡					Uptake of [^{14}C]DXR§	
Cell line	DXR	Act D	VBL	VCR	5-FU	2 hr	4 hr
MCF-7 WT	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MCF-7^{adr}	7.3‖	15.0‖	8.2‖	10.0‖	1.0	2.2‖	2.5‖
CT-26P	2.0	1.5	2.1	2.2	1.0	1.0	1.0
CT-26 R500	5.1‖	7.0‖	4.1‖	5.0‖	1.0	2.1‖	2.1‖

* Enhancement ratio (IC_{50}) is the IC_{50} of a drug in the drug-treated cells/IC_{50} of the same drug in the drug- and CGP 41251-treated cells. The carrier for active CGP 41251 had no effect.

† Enhancement ratio (uptake) is the uptake of [^{14}C]DXR in the presence of CGP 41251/the uptake of [^{14}C]DXR in the absence of CGP 41251. The carrier for active CGP 41251 had no effect.

‡ Tumor cells were seeded at 1–3 × 10^3 cells/well in 96-well tissue culture plates. After an attachment period of 18 hr, quadruplicate samples were incubated for 4 days with a dose range of the standard drugs with or without CGP 41251. Abbreviations not defined previously: VBL, vinblastine; VCR, vincristine; and Act D, actinomycin D. Cytostasis was determined by the MTT assay. The carrier for active CGP 41251 had no effect. Results are representative of at least two experiments done in quadruplicate.

§ Cells were seeded at 0.5 × 10^6 cells/dish in 35-mm tissue culture dishes for 18 hr. Fresh medium with or without CGP 41251 and [^{14}C]DXR were added to the cultures. At various times, the medium was removed, cells were washed, and the intracellular radioactivity was monitored. The carrier for active CGP 41251 had no effect. Results are representative of at least three experiments done in duplicate.

‖ $P < 0.05$, compared with medium only.

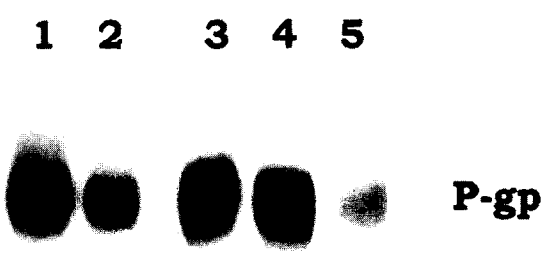

FIG. 2. Decreased phosphorylation of P-gp in cells treated with CGP 41251. MCF-7^{adr} and CT-26 R500 cells were pretreated with CGP 41251 for 1 hr and incubated in phosphate-free medium in the presence of [^{32}P]inorganic phosphate and CGP 41251 for another hour. Total protein was collected, and P-gp was immunoprecipitated with 5 μg of C219 anti-P-gp monoclonal antibody from 200 μg of protein. The immune complexes were isolated using protein-A-Sepharose beads, and the resulting sample was run on an SDS–PAGE gel. The gel was fixed, stained, and exposed for 72 hr at -70° using Hyperfilm-MP. Key: MCF-7^{adr} (lane 1) with CGP 41251 at 125 nM (lane 2), CT-26 R500 (lane 3) with CGP 41251 at 30 nM (lane 4), and with CGP 41251 at 310 nM (lane 5).

reduction in P-gp phosphorylation in MCF-7^{adr} cells treated with CGP 41251. In the CT-26 R500 cells, a higher concentration (310 nM) was required to reduce P-gp phosphorylation by 80% (Fig. 2).

In summary, we have shown that nontoxic concentrations of CGP 41251 sensitized MDR cells to MDR-related drugs but not to 5-FU. This partial reversal of the MDR phenotype was mediated by an increased intracellular accumulation of the chemotherapeutic agents, which was associated with a decrease in P-gp phosphorylation. Our results support a mechanism of MDR reversal by CGP 41251 that entails inhibition of the phosphorylation of P-gp and/or other PKC substrates critical to the MDR phenotype rather than competitive binding to the drug-efflux pump and that may circumvent the toxicity associated with classical P-gp-binding MDR reversal agents. In fact, we have already demonstrated the efficacy of CGP 41251 in the reversal of drug resistance in metastatic tumors in an *in vivo* nude mouse model [19], indicating the potential significance of its novel mechanism of MDR reversal.

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References

1. Juliano RL and Ling V, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455:** 152–162, 1976.
2. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948:** 87–128, 1988.
3. Fan D, Beltran PJ and O'Brian CA, Reversal of multidrug resistance. In: *Reversal of Multidrug Resistance in Cancer* (Ed. Kellen JA), pp. 93–125. CRC Press, Boca Raton, 1994.
4. O'Brian CA, Fan D, Ward N, Seid C and Fidler IJ, Level of protein kinase C activity correlates directly with resistance to adriamycin in murine fibrosarcoma cells. *FEBS Lett* **246:** 78–82, 1989.
5. Posada JA, McKeegan EM, Worthington KF, Morin MJ, Jaken S and Tritton TR, Human multidrug resistant KB cells overexpress protein kinase C: Involvement in drug resistance. *Cancer Commun* **1:** 285–292, 1989.
6. Gravitt KR, Ward NE, Fan D, Skibber JM, Levin B and O'Brian CA, Evidence that protein kinase C-α activation is a critical event in phorbol ester-induced multidrug resistance in human colon cancer cells. *Biochem Pharmacol* **48:** 375–381, 1994.
7. Yu G, Ahmad S, Aquino A, Fairchild CR, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Cowan KH and Glazer RI, Transfection with protein kinase C-α confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. *Cancer Commun* **3:** 181–189, 1991.
8. Bates SE, Lee JS, Dickstein B, Spolyar M and Fojo AT, Differential modulation of P-glycoprotein transport by protein kinase inhibition. *Biochemistry* **32:** 9156–9164, 1993.
9. Blobe GC, Sachs CW, Khan WA, Fabbro D, Stabel S, Wetsel WC, Obeid LM, Fine RL and Hannun YA, Selective regulation of expression of protein kinase C isoenzymes in multidrug-resistance MCF-7 cells. Functional significance of enhanced expression of PKC-α. *J Biol Chem* **268:** 658–664, 1993.
10. Ma LD, Marquardt D, Takemoto L and Center MS, Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J Biol Chem* **266:** 5593–5599, 1991.
11. Meyer T, Rehenass U, Fabbro D, Alteri E, Rosel J, Muller M, Carvatti G and Matter A, A derivative of staurosporine (CGP 41251) shows selectivity for PKC inhibition and *in vitro* antiproliferative as well as *in vivo* antitumor activity. *Int J Cancer* **43:** 851–856, 1989.
12. Andrejauskas-Buchdunger E and Regenass U, Differential inhibition of the epidermal growth factor-, platelet-derived growth factor-, and protein kinase C-mediated signal transduction pathways by the staurosporine derivative CGP 41251. *Cancer Res* **52:** 5353–5385, 1992.
13. Utz I, Hofer S, Regenass U, Hilbe W, Thaler J, Grunicke H and Hofmann J, The protein kinase C inhibitor CGP 41251, a staurosporine derivative with antitumor activity, reverses multidrug resistance. *Int J Cancer* **57:** 104–110, 1994.
14. Corbett TH, Griswold DP Jr, Roberts BJ, Peckman JC and Schabel FM Jr, Tumor induction relationships in developing transplantable cancers of the colon in mice for chemotherapeutic assays with a note on carcinogen structure. *Cancer Res* **35:** 2434–2439, 1975.
15. Fan D, Poste G, Ruffolo RR Jr, Dong Z, Seid C, Earnest LE, Campbell TE, Clyne RK, Beltran PJ and Fidler IJ, Circumvention of multidrug resistance in murine fibrosarcoma and colon carcinoma cells by treatment with the α-adrenoceptor antagonist furobenzazepine. *Int J Oncol* **4:** 789–798, 1994.
16. Budworth J, Davies R, Malkhandi J, Gant TW, Ferry DR and Gescher A, Comparison of staurosporine and four analogues: Their effects on growth, rhodamine 123 retention and binding to P-glycoprotein in multidrug-resistant MCF-7/Adr cells. *Br J Cancer* **73:** 1063–1068, 1996.
17. Sampson E, Wolff CL and Abraham I, Staurosporine reduces P-glycoprotein expression and modulates multidrug resistance. *Cancer Lett* **68:** 7–14, 1993.
18. Chaudhary PM and Roninson IB, Activation of MDR1 (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncol Res* **4:** 281–290, 1992.
19. Killion JJ, Beltran P, O'Brian CA, Yoon S-S, Fan D, Wilson MR and Fidler IJ, The antitumor activity of doxorubicin against drug-resistant murine carcinoma is enhanced by oral administration of a synthetic staurosporine analogue, CGP 41251. *Oncol Res* **7:** 453–459, 1995.