Anti-Cancer Drugs 1995, **6**, pp. 70–76

Effects of protein kinase C inhibitor, staurosporine derivative CGP 41 251, on cell cycle, DNA synthesis and drug uptake in neoplastic cell lines

Jan Sedlak, Luba Hunakova, Jozef Duraj, Branko Chorvath and Ladislav Novotny
Cancer Research Institute, Slovak Academy of Sciences, Spitalska 21, 812 32 Bratislava, Slovakia.
Tel: (+42) 7 349 331; Fax: (+42) 7 326 140.

The protein kinase C inhibitor, staurosporine derivative CGP 41 251, was more efficient than staurosporine in the reversal of decreased anthracycline uptake in the anthracycline-resistant cell subline (A2780/ADR) of ovarian carcinoma. Staurosporine was more efficient than CGP 41 251 in the induction of cytometrically determined DNA fragmentation (cytofluorometric equivalent of apoptosis) in A2780 parental human ovarian carcino-ma cells compared with the drug-resistant A2780/ADR subline and in both human leukemia K-562 cells as well as mouse leukemia L1210 compared with the araC-resistant L1210 cells. Staurosporine was a more potent inhibitor than CGP 41 251 of DNA synthesis in both araC-sensitive and -resistant mouse leukemia L1210 cells. CGP 41 251 was a slightly more efficient inhibitor of thymidine incorporation than staurosporine in human leukemia K-562 cells and its combination with araC had a higher inhibitory effect on the DNA synthesis in this cell line than staurosporine. CGP 41 251 exerted DNA synthesis inhibitory effects on both araC-sensitive and -resistant L1210 cells. Staurosporine-induced DNA synthesis inhibition in both araC-resistant and -sensitive L1210 mouse leukemia cells was decreased after combined administration with araC.

Key words: Cell cycle, CGP 41 251, DNA synthesis, drug resistance, flow cytometry, K-562 leukemia cells, L1210 mouse leukemia, ovarian carcinoma A2780 cells, protein kinase inhibitors, staurosporine.

Introduction

Protein kinase C (PKC) inhibitors, such as staurosporine (a microbial alkaloid produced by a strain of *Streptomyces*), were shown to affect control of cytokine-induced and 12-*O*-tetradecanoyl-13-phorbol acetate (TPA)-stimulated cell surface antigen modulation,[1] and to modify the regulation of functions related to some such cell surface molecules.[2] Staurosporine was shown to affect the cell cycle of normal and neoplastic cells,[3] and was instrumental in studies leading to the conclusion that multiple kinase mediated phosphorylations of various cellular substrates are involved in the progression of cells through the G_1 phase of the cell cycle.[4] Staurosporine appeared also to modulate (decrease) the efficiency of the *mdr1* (gp 170) drug efflux system.[5–7]

A new derivative of staurosporine, CGP 41 251,[8] was recently shown to be a more selective inhibitor of PKC, with a significant anti-tumor activity *in vivo* and lower cytotoxicity.[9] Recent reports demonstrated the effects of CGP 41 251 on epidermal growth factor- and platelet derived growth factor-elicited, PKC-mediated cell membrane signal transduction[9] and neoplastic (Walker carcinosarcoma) cell motility.[10] Our report describe preliminary data on comparison of CGP 41 251 and staurosporine effects on drug uptake in a drug-resistance ovarian carcinoma cell line, as well as cell cycle alterations induced by staurosporine or CGP 41 251 in drug-sensitive and -resistant sublines of human ovarian A2780 cells and mouse leukemia L1210 cells. Furthermore, different effects of staurosporine and CGP 41 251, and their interference with araC on thymidine uptake in araC -resistant mouse leukemia L1210 cells and human K-562 leukemia line are reported.

Materials and methods

Cell lines

Human myeloid-erythroid leukemia cell line K-562 was cultured as a suspension culture in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum, in a 5.0% CO_2 humidified atmosphere. Anthracycline-sensitive and -resistant sublines of A2780 ovarian carcinoma cells were obtained from Dr A McGown (Patterson Institue, Manchester, UK) and were cultured in MEM cell

This study was supported by the grants 1335/94 and 1331/94 from the Grant Agency for Sciences, Slovakia

Correspondence to B Chorvath

culture medium under identical conditions. Characterization of anthracycline uptake in A2780/ADR cells and their surface antigen phenotype were described previously.[11] The murine leukemia cell line L1210 was originally obtained from the Institute for Cancer Research (Professor K Harrap), Sutton, Surrey, UK. The murine araC-resistant leukemia cell line L1210/araC was derived from the parental L1210 cell line as described previously.[12] The L1210/S (araC-sensitive) and L1210/araC (resistant) cell lines were maintained *in vivo* using inbred DBA/2J mice of both sexes weighing 18–20 g (Velaz, Prague, Czech Republic). The animals were reared under standard conditions and kept in groups of 10. The transplantation of L1210/S and L1210/araC cells was performed every fifth day after inoculation by i.p. administration of 10^6 leukemia cells per mouse.

Isolation of cells from ascitic fluid

L1210/S and L1210/araC cells were isolated from the ascitic fluid by centrifugation on 17% Verografin (Spofa, Prague, Czech Republic), washed twice with 0.9% sodium chloride and resuspended in RPMI 1640 cell culture medium.

Compounds

Staurosporine was from Sigma (St Louis, MO). CGP 41 251 was obtained by the courtesy of CIBA-GEIGY (Basel, Switzerland) and was dissolved in dimethyl-sulfoxide, according to the instructions of the producer. Phorbol ester TPA was from Sigma. Arabinosylcytosine (araC, NSC 287 459) was prepared as described elsewhere (13). The water used for the preparation of buffers and solutions was purified by passing deionized water through an Elgastat Spectrum SD 20 (Elga, High Wycombe, UK). All other chemicals were of the highest purity available.

Cell cycle analyis and flow cytometric determination of anthracyline content

FACS analysis were performed with the aid of FACStar (Becton Dickenson Mountain View, CA) flow cytometer equipped with a 5 W argon ion laser tuned to 488 nm excitation wavelength, according to the instructions of the producer. Evaluation of flow cytometric measurements was performed with the PC-Lysys software provided by the producer (Becton Dickinson). The significance of differences in anthracycline content modulation of A2780/ADR cells were evaluated with the aid of Kolmogorov–Smirnov statistics.[14] The differences in daunomycin uptake (FL2) histograms of staurosproine- or CGP 41 251-treated versus untreated cells, expressed as the Kolmogorov–Smirnov $D/(s)$ values, reflected the efficiencies of drug uptake modulation by both protein kinase inhibitors (staurosporine and CGP 41 251).

DNA synthesis

The activity of DNA synthesis was determined by 6-[^{3}H]thymidine with a specific activity of 980 GBq/mol (Institute for Research, Development and Production of Radionuclides, Prague, Czech Republic). Cells were incubated in the presence of radiolabeled precursor for 10 min (185 kBq/ml and 5×10^6 cells/ml). The incorporation of radionuclides into the trichloroacetic acid-insoluble cell fraction was measured with the aid of a Packard TriCarb liquid scintillation counter.

Results

Effect of protein kinase inhibitors (staurosporine and CGP 41 251) on anthracycline uptake in A2780/ADR cells

The anthracyline-resistant subline of the A2780 ovarian carcinoma cell line displayed two peaks of anthracycline (red fluorescence)-containing cells (Figure 1A), corresponding apparently to two cell subpopulations with different anthracycline uptake–efflux characteristics, both of these subpopulations viable at 300 nM daunomycin (i.e. two anthracycline-resistant subpopulations). The peak with the lower intensity of red fluorescence (absent in the parental anthracycline-sensitive A2780 cell line; data not shown) was quantitatively reduced in the resistance subline after treatment of cells by the conventional *mdr1* drug resistance modulator verapamil (Figure 1D). Both staurosporine and mainly CGP 41 251 produced a verapamil-like effect in the resistant A2780 subline (Figure 1B and C). The maximal difference between the drug resistance modulation efficiency of CGP 41 251 versus staurosporine corresponded to approximately 20–30 times higher CGP 41 251 drug uptake-modulating activity in the concentration

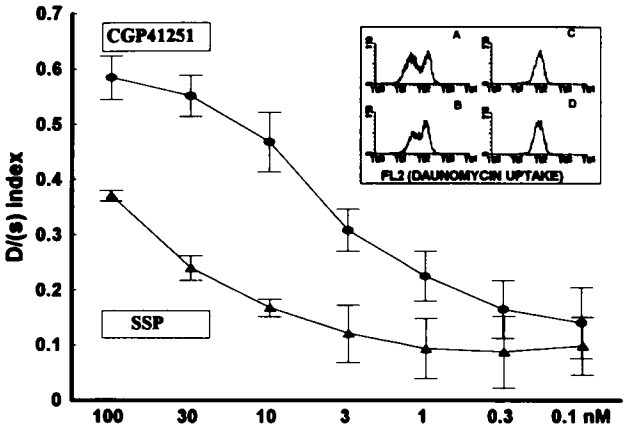

Figure 1. Comparison of daunomycin content modulating efficiencies (CGP 41 251 versus staurosporine) in the A2780/ADR drug-resistant ovarian carcinoma cell line. CGP 41 251 (circles), staurosporine (triangles) concentration at the abscissa. Ordinate: Komogorov–Smirnov *D/(s)* value representing the difference in daunomycin content of CGP 41 251- or staurosporine-treated versus untreated (control) A2780/ADR cells. Insert shows the bimodal curve of daunomycin cell content of A2780/ADR cell (A), a slight decrease of low daunomycin content peak after treatment with 30 nM staurosporine (B) and complete abrogation of this peak after treatment with 30 nM CGP 41 251 (C) or 10 μM verapamil (D).

range of 1–100 nM for both protein kinase inhibitors (Figure 1).

Cell cycle alterations induced by staurosporine and CGP 41 251 in K-562 and A2780/ADR cells

Staurosporine modified the cell cycle of the human immature leukemia cell line K-562, with the accumulation of cells with a higher than G_2/M phase DNA cell content and also caused DNA histogram alterations characteristic of apoptosis (i.e. the accumulation of cells and cell debris in the region of the DNA histogram to left of the G_0/G_1 peak) (Figure 2). On the other hand, CGP 41 251 was more efficient in inducing accumulation of cells of both A2780 sublines with a higher than G_2/M cell cycle phase DNA content. Both L1210 and A2780 drug-resistant sublines appeared more resistant to the apoptotic changes of the DNA histograms. In the A2780/ADR ovary carcinoma cells, CGP 41 251 was more efficient than staurosporine in induction of both cell cycle alteration (accumulation in G_2/M phase) and modulation of decreased anthracycline cell content (Figure 2).

The effect of protein kinase inhibitors (staurosporine and CGP 41 251) on DNA synthesis in leukemia cell lines

The pattern of DNA synthesis inhibition by staurosporine, CGP 41 251 and their combinations with araC differed between the cell lines examined. In K-562 cells the inhibition of DNA synthesis induced by araC was similar to that caused by CGP 41 251, while staurosporine exerted a lower DNA synthesis inhibitory effect. In these cells, CGP 41 251 enhanced the inhibition of DNA synthesis induced by araC (Table 1). In both L1210/S and L1210/araC cells, staurosporine at the utilized concentration appeared as a more potent inhibitor of DNA synthesis than CGP 41 251. Simultaneous use of araC and staurosporine resulted in DNA synthesis inhibition lower than that caused by staurosporine alone in L1210 araC-resistant mouse leukemia cells (Tables 2 and 3).

Discussion

Protein kinases are involved in the regulation of normal and neoplastic cell proliferation, differentiation and cell membrane signal transduction. PKC was shown to play a key role[15] in signal transduction pathways, elicited by such cell surface ligands as growth factors, cytokines, neurotransmitters, hormones, etc. PKC inhibitors have been instrumental in the elucidation of molecular mechanisms of cell membrane signal transduction.

In our study we used a benzoylated staurosporine derivative CGP 41 251, that was shown to be a more selective, although less potent PKC inhibitor.[8] The reversal of multidrug resistance by PKC inhibitor CGP 41 251 was recently demonstrated in a human multidrug resistant lymphoblastoid cell line.[16] Our data extend this observation on a human ovarian carcinoma drug resistant cell line. CGP 41 251 was, on an equimolar basis, a significantly more potent modulator of anthracycline uptake–efflux in an anthracycline-resistant subline of ovarian carcinoma cells. This result suggests a predominant role of PKC in modulation of verapamil-sensitive anthracycline uptake–efflux in the examined drug-resistant A2780 ovarian carcinoma cell subline. The broader and more complex profile of protein kinase inhibitory activity exerted by staurosporine might mitigate the PKC inhibitory effect of staurosporine, resulting in lower anthracycline uptake-modulating activity as compared with a more selective PKC inhibitor (i.e. CGP 41 251).

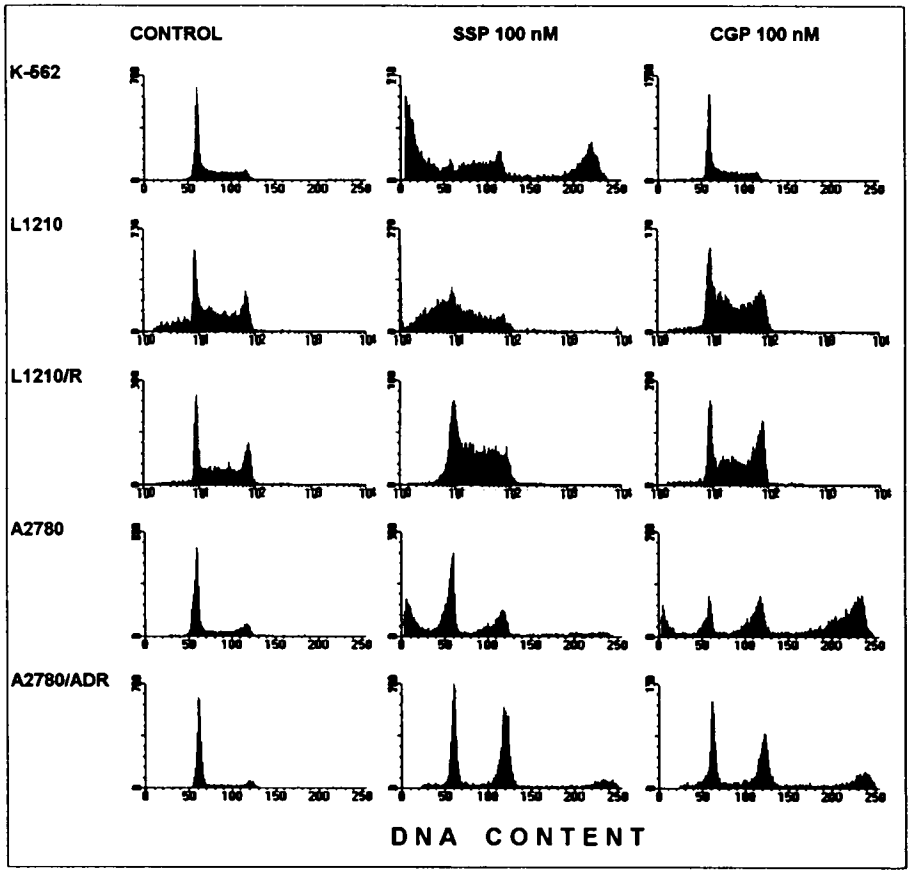

Figure 2. Flow cytometric DNA content histograms of human leukemia cell line K-562, mouse leukemia L1210, its araC-resistant subline (L1210/R), human ovarian carcinoma A2780 and its anthracycline-resistant subline, after treatment with equimolar (100 nM) concentrations of staurosporine or CGP 41 251. Ordinate: relative number of cells.

Table 1. Inhibition of DNA synthesis by staurosporine (SSP) and CGP 41 251 in K-562 (5×10^5 cells/ml) leukemia cells

	Treatment			DNA synthesis (% ± SD)	Significance ($p<$)			Expected synthesis of DNA (%)	*R*
	araC (μM)	SSP (nM)	CGP (nM)						
1	—	—	—	100 ± 2.8					
2	10	—	—	33 ± 2.1			*		
3	—	400	—	43 ± 7.0	*				
4	—	100	—	64 ± 2.1		*			
5	10	400	—	30 ± 2.1	0.05		NS	14	2.1
6	10	100	—	21 ± 3.1		0.001	NS	21	1.0
7	—		400	34 ± 1.2	*				
8	—		100	54 ± 3.5		*			
9	10		400	16 ± 1.0	0.0001		0.01	11	1.4
10	10		100	15 ± 0.5		0.0001	0.005	18	0.8

NS, not significant. * Significance calculated in relation to this group. *R*, ratio of DNA synthesis (% of control) experimental/expected.

Recently, different effects of staurosporine on the cell cycle of normal and leukemic lymphocytes was described. Staurosporine arrested MOLT4 cells in G_2/M phase of the cell cycle.[17] The use of three neoplastic cell lines (mouse and human leukemia, human carcinoma, among them two resistant pairs) allowed us to widen the spectrum of different effects of staurosporine and CGP 41 251 inhibitors. Accumulation of the cells with higher than G_2/M cell cycle phase DNA content (i.e. possibly the induction of multinuclear cells) induced by CGP 41 251 could be seen only in human carcinoma cells.

Table 2. Inhibition of DNA synthesis by staurosporine (SSP) and CGP 41 251 in L1210/S (5×10^5 cells/ml *in vitro*) leukemia cells

	Treatment			DNA synthesis (% ± SD)	Significance ($p<$)			Expected synthesis of DNA (%)	*R*
	araC (μM)	SSP (nM)	CGP (nM)						
1	—	—	—	100 ± 5.6					
2	0.05	—	—	49 ± 4.6			*		
3	—	400	—	20 ± 0.4	*				
4	—	100	—	36 ± 4.9		*			
5	0.05	400	—	30 ± 3.0	0.08		0.002	10	3.0
6	0.05	100	—	46 ± 1.7		0.07	NS	18	2.6
7	—		400	73 ± 3.5	*				
8	—		100	84 ± 5.7		*			
9	0.05		400	64 ± 3.0	0.02		0.005	36	1.8
10	0.05		100	60 ± 2.0		0.001	0.01	41	1.5

See Table 1.

Table 3. Inhibition of DNA synthesis by staurosporine (SSP) and CGP 41 251 in L1210/araC (5×10^5 cells/ml *in vitro*) leukemia cells

	Treatment			DNA synthesis (% ± SD)	Significance ($p<$)			Expected synthesis of DNA (%)	*R*
	araC (μM)	SSP (nM)	CGP (nM)						
1	—	—	—	100 ± 4.7					
2	10	—	—	88 ± 1.9			*		
3	—	400	—	46 ± 0.3	*				
4	—	100	—	42 ± 2.3		*			
5	10	400	—	61 ± 5.5	0.005		0.001	41	1.5
6	10	100	—	59 ± 3.0		0.001	0.0003	37	1.6
7	—		400	76 ± 6.0	*				
8	—		100	98 ± 4.7		*			
9	10		400	69 ± 2.5	NS		0.001	67	1.0
10	10		100	91 ± 2.8		NS	NS	86	1.1

See Table 1.

On the other hand, staurosporine-induced changes in the DNA histogram pattern typical for apoptotic cells were accompanied by multinuclear cells in K-562 and to a lesser extent also in A2780 cells. The concentration-dependent efficiency of staurosporine and CGP 41 251 in both cell cycle alterations and anthracycline content modulations revealed a reverse order: 10 nM concentration of staurosporine produced the accumulation of K-562 cells in G_2/M phase. This type of cell cycle alteration was observed at 800 nM CGP 41 251 (data not shown). On the other hand, CGP 41 251 was more efficient than staurosporine in the reversal of decreased anthracycline cell content and in the induction of cell cycle alterations in A2780/ADR cells.

The leukemia cell lines chosen for these experiments are known for their various sensitivities to araC, a drug requiring phosphorylation to its triphosphate (araCTP) prior to its cytostatic action on intracellular target structures. The murine leukemia L1210/S cells phosphorylate araC using deoxycytidine kinase, the crucial enzyme in araC metabolism. The phosphorylation of araC in this cell line is relatively rapid and the L1210/S leukemia line is highly sensitive to araC. On the other hand, the L1210/araC murine leukemia line possesses a very low sensitivity to araC. The K-562 leukemia cell line derived from a patient with chronic myelogenous leukemia has an intermediate sensitivity to araC—a cytostatic drug used in medical oncology. The results are compatible with the previously described drug and cell line characteristics.

The cytotoxicity of staurosporine and CGP 41 251 administered independently (as a sole cytotoxic agent) to the examined leukemia lines was dose dependent. Both compounds, staurosporine and

CGP 41 251, known to inhibit protein kinase activity, also interfered with the biological activity (inhibition of DNA synthesis) of araC in the leukemia lines examined. This inhibition of DNA synthesis changed in a dose-dependent manner for both the tested combinations of compounds, i.e. staurosporine–araC and CGP 41 251–araC. These data might suggest a possible interference of the studied protein kinase inhibitors with araC phosphorylation or with other sites of molecular pathway(s) required for araC-mediated DNA synthesis inhibition. The highest level of staurosporine interference with DNA synthesis inhibition caused by araC was found in L1210/araC cells and the lowest in L1210/S cells. This is obvious when DNA synthesis determined in our experiments is compared with that calculated as expected for an additive effect, from the inhibiton of DNA synthesis caused by araC and staurosporine or CGP41 251 utilized independently, i.e. as the sole cytostatic agent (data in column *R* of the tables).

CGP 41 251 in our experiments did not reverse the resistance to araC, which is not caused by the multidrug resitance mechanism (i.e. enhanced drug efflux due to the activity of p170 *mdr* glycoprotein). Our data corroborate the findings that the CGP 41 251 inhibitory activity is more specifically directed towards PKC, as this compound interfered with araC DNA inhibitory activity less efficiently than staurosporine. Consequently, our results suggest that staurosporine might interact with a broader range of cellular kinases, including the one(s) involved in araC phosphorylation.

Studies of selective PKC inhibitors and their ability to reverse *mdr1* gene drug resistance show the efficiency of such staurosporine derivatives (NA-382, UCN-01) in the modulation of drug resistance in several neoplastic cell lines.[18–20] Selective PKC inhibitors including CGP 41 251 appear to be a useful laboratory tool for elucidation of neoplastic cell proliferation, differentiation, signal transduction and for the evaluation of possibilities to modulate drug uptake–efflux kinetics in drug-resistant neoplastic cells.

Conclusions

The PKC inhibitor CGP 41 251 reversed the decreased daunomycin uptake in a drug-resistant subline of the ovarian carcinoma cells A2780 *in vitro*. CGP 41 251 effectively induced the accumulation of apoptotic A2780 cells and L1210/S mouse leukemia cells in the 'pre-G_0/G_1 region' of DNA histograms. CGP 41 251 enhanced the DNA synthesis inhibition induced by araC in human K-562 leukemic cells *in vitro*.

Acknowledgements

Authors express their thanks to CIBA-GEIGY (Basel, Switzerland) for the CGP 41 251 inhibitor. The excellent technical cooperation of Mrs M Sulikova, D Pailingerová and G Gasajova is appreciated.

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(Received 12 July 1994; accepted 18 August 1994)