Staurosporine increases toxicity of gemcitabine in non-small cell lung cancer cells: role of protein kinase C, deoxycytidine kinase and ribonucleotide reductase

Jennifer Sigmond, Andries M. Bergman, Leticia G. Leon, Willem J.P. Loves, Eveline K. Hoebe and Godefridus J. Peters

Gemcitabine, a deoxycytidine analog, active against non-small cell lung cancer, is phosphorylated by deoxycytidine kinase (dCK) to active nucleotides. Earlier, we found increased sensitivity to gemcitabine in P-glycoprotein (SW-2R160) and multidrug resistance-associated protein (SW-2R120), overexpressing variants of the human SW1573 non-small cell lung cancer cells. This was related to increased dCK activity. As protein kinase C (PKC) is higher in 2R120 and 2R160 cells and may control the dCK activity, we investigated whether gemcitabine sensitivity was affected by the protein kinase C inhibitor, staurosporine, which also modulates the cell cycle. Ten nmol/l staurosporine enhanced the sensitivity of SW1573, 2R120 and 2R160 cells 10-fold, 50-fold and 270-fold, respectively. Staurosporine increased dCK activity about two-fold and the activity of thymidine kinase 2, which may also activate gemcitabine. Staurosporine also directly increased dCK in cell free extracts. Staurosporine decreased expression of the free transcription factor E2F and of ribonucleotide reductase (RNR), a target for gemcitabine inhibition. In conclusion, staurosporine may potentiate gemcitabine by increasing dCK and decreasing E2F and RNR, which will lead to a more pronounced RNR inhibition. *Anti-Cancer Drugs* 21:591–599 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

2′,2′-Difluorodeoxycytidine (dFdC, gemcitabine) is a deoxycytidine (dCyd) analog, clinically active against non-small cell lung cancer (NSCLC), in line with its activity against in-vitro and in-vivo solid tumor models [1–3]. After cellular uptake, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) and, to a lesser extent, by the mitochondrial enzyme thymidine kinase 2 (TK2) [4,5]. The gemcitabine phosphates are the active metabolites. Triphosphate dFdCTP can be incorporated into DNA and RNA for which it competes with deoxycytidine triphosphate (dCTP) [6,7]. As dCTP is the major natural feedback inhibitor of dCK and competes with dFdCTP for DNA polymerase, a decrease in the dCTP pools will increase gemcitabine sensitivity [8]. Diphosphate dFdCDP inhibits ribonucleotide reductase (RNR) [8], which is responsible for catalyzing the reduction of ribonucleotides to their corresponding deoxyribonucleotides essential for DNA synthesis and repair of DNA damage. RNR is a tetramer consisting of two nonidentical homodimers. The two identical M2 subunits regulate the substrate specificity of the enzyme, whereas the other two identical M1 subunits are responsible for the activity by binding the ribonucleotides and allosteric effectors [9,10].

Protein kinase C (PKC) is a family of enzymes that are physiologically activated by 1,2-diacylglycerol and other lipids, which play a role in signal transduction, thereby controlling several pathways such as cell cycle progression [11]. Wang *et al.* described an effective phosphorylation of dCK, associated with a two-fold increase in the V_{max}. The investigators suggested that the phosphorylation of dCK is catalyzed by PKC [12]. However, recently Smal *et al.* described that dCK can be phosphorylated at the Ser-74 site, but that PKC was not involved. This phosphorylated form had a higher activity [13].

Overexpression of the membrane efflux pumps, P-glycoprotein (P-gP) and multidrug resistance-associated protein (MRP), is associated with a broad cross-resistance phenotype [14,15]. In our earlier study an increased sensitivity to gemcitabine was found in various couples of MRP and P-gP overexpressing cells as compared with

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their parental cells [16]. Variants of the human NSCLC cell line SW1573, 2R120 and 2R160, were used, which overexpressed MRP and P-gP, respectively. Results of this study showed that gemcitabine sensitivity increased nine-fold and 28-fold in 2R120 and 2R160, respectively as compared with their parental cell line. This collateral sensitivity was related to a seven-fold and four-fold increase in dCK activity in 2R120 and 2R160 cells, respectively, which was associated with an increase in dCK mRNA and dCK protein. Several studies report an increase in PKC activity in cells with a P-gP or MRP overexpression [17,18]. It is known that P-gP and MRP can be phosphorylated [19,20], but whether the phosphorylation modulates the pump function is a matter of debate [18–21]. Staurosporine (STS) and its derivatives are PKC inhibitors, which can reverse the MDR phenotype resulting in increased drug accumulation, possibly by modulating the phosphorylation of P-gP and MRP [22–24].

Inhibition of PKC modulates the efficacy of treatment with various cytostatic agents. Noncytotoxic concentrations of the more specific STS derivative CGP41251 (benzoylstaurosporine) increased the sensitivity to doxorubicin in the MDR variants of murine carcinoma cell lines [25], and that of doxorubicin, actinomycin D, vinblastin and vincristine in the MDR variants of MCF-7 human breast carcinoma cells and CT-26 murine colon adenocarcinoma cells [24]. A synergistic toxicity between PKC inhibitors and deoxynucleoside analogs has also been described in several studies, both in solid and hematological malignancies [26–29]. Although STS is a potent PKC inhibitor, it has several other effects on cellular functions such as modulation of cell cycle progression. These effects can also be achieved at lower concentrations, although it has also been shown that cell cycle arrest may affect transcription factors such as E2F, influencing the activity of the key enzyme in gemcitabine metabolism, dCK.

We investigated whether STS can affect sensitivity to gemcitabine, and whether this is mediated by the inhibition of PKC or whether STS mediated effects on the cell cycle would lead to modulation of the gemcitabine activating enzyme, dCK.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium was purchased from Flow Laboratories (Irvine, UK) and fetal calf serum (FCS) from Gibco (New York, USA), trichloroacetic acid, glutamine and gentamicin from Merck (Darmstadt, Germany), trypsin, sulforhodamine B (SRB) and STS from Sigma Chemical Co (St Louis, USA), and [5–^{3}H]-deoxycytidine (21.9 Ci/mmol) from Moravek, Brea, Ca. The PKC antibody (MC5, Mouse monoclonal IgG2a) was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). The antibodies directed to the M2 subunit of RNR (clone N-18/I-15/E-16/H-300) and to E2F1 (clone KH-95) were from Santa Cruz Biotechnologies. Lipofectamine 2000 was obtained from Invitrogen life technologies (Breda, The Netherlands). Locked nucleic acid (LNA) for PKC-α (registered as Cur2053; sequence 5′-AAAACGTCAGCCATGGTCCC-3′) was purchased from Exiqon (Vedbaek, Denmark). All other chemicals were of analytical grade and commercially available.

Cell culture

The in-vitro experiments were performed with the human NSCLC cell line H292, A549, H460 and SW1573 and its doxorubicin resistant variants 2R120 (overexpressing MRP), 2R160 (overexpressing P-gP), which were grown in monolayers in Dulbecco's modified Eagle's medium at 37°C and 5% CO_2, supplemented with 7.5% heat inactivated FCS and 250 ng/ml gentamicin [30,31]. The cells were regularly screened for Mycoplasma contamination by using a rapid detection system with a ^{3}H-labelled DNA probe (Gen-Probe, San Diego, Californiia, USA) and were found to be negative.

Chemosensitivity testing

The determination of the IC-50 (the drug concentration causing 50% growth inhibition) in the monolayer cell lines was performed using the SRB assay. The assay was performed using the NCI protocol with some small modifications [32,33]. Culture conditions were optimized for all cell lines and the cell size of the cells was microscopically checked on a regular basis during exposure to gemcitabine and STS. At day 1, the cells were plated in 96-well plates in different densities. The optimal plating number was the highest number of cells possible to enable a log linear growth for 96 h (SW1573 8000; 2R120 15 000; and 2R160 15 000 cells/well) in a volume of 100 µl per well. On day 2, drugs were added to the wells to a final concentration range (5×10^{-16} to 5×10^{-5} mol/l) of gemcitabine and a fixed concentration of 0, 5, 10 or 50 nmol/l STS. After 72 h of drug exposure, the cells were precipitated with 50 µl ice-cold 50% w/v trichloroacetic acid, after which the SRB assay was performed. The optical density (OD) was measured at 540 nm. Growth inhibition curves consisted of the relative to control ODs of every SRB assay. The points were connected by straight lines and the IC_{50} values (relative OD at 50%) were determined from the interpolated graph [34]. A relative growth (RG) of 0% indicates a total growth inhibition and lower than 0% indicates a cell kill.

Interaction between STS and gemcitabine was evaluated by comparing the RG of the cells exposed to gemcitabine alone with the RG of those exposed to the combination of gemcitabine and STS, according to the fractional product method of Webb [35,36]. For this purpose, we compared the RG of the combination at the concentration of gemcitabine alone, which gave a 50% growth inhibition (RG: 0.5). We calculated the expected growth of gemcitabine with STS by multiplying 0.5 with the RG of STS

alone. This was compared with the measured RG of gemcitabine and STS. At the STS concentrations of 5, 10 or 50 nmol/l, a ratio was calculated of the measured and expected RG (M/E ratio).

$$\text{M/E ratio} = \frac{RG_{\text{combination STS and gemcitabine (at } IC_{50} \text{ concentration)}}}{RG_{STS} \times RG_{\text{gemcitabine (at } IC_{50} \text{ concentration)}} (= 0.5)}$$

A ratio greater than 1.1 indicates an antagonistic interaction, a ratio of 0.9–1.1 indicates additivity and a ratio less than 1.1 indicates a synergistic interaction between gemcitabine and STS. Differences between the measured and expected growth inhibition were evaluated by a *t*-test. This method of analyzing drug interactions allowed us to study the combination of gemcitabine at a concentration range and STS at a fixed concentration. Moreover, data on cell kill ($RG \leq 0$) could be used to evaluate the drug interaction [37], which would not have been possible with the multiple drug effect analysis as in this method values below 0 (fraction affected > 1.0) cannot be evaluated. Results were also analyzed by the isobologram analysis [36,38]; a point on the x-axis represents a STS concentration, a point on the y-axis represents a gemcitabine concentration inducing the same growth inhibition as the STS concentration. A line drawn between these points represents all the concentrations of the combinations of gemcitabine and STS inducing the same growth inhibition as either drug separately and thus, represents additivity (calculated). An observed position (measured) to the left of this line indicates synergism and to the right it indicates antagonism [38].

Enzyme activities

For determination of the effect of STS on dCK and TK2 activities in whole cells, the cells were cultured in a medium containing 0, 5, 10 or 50 nmol/l STS. We earlier determined that PKCα could be inhibited under both the conditions [39]. After a 24 h culture (with or without STS), the cells were harvested, lysed and 10 000 g supernatants were prepared in a 0.3 mol/l Tris-HCl and 50 µmol/l β-mercaptoethanol (to stabilize the enzyme) containing buffer (pH 8.0) essentially as described [40]. Protein content was estimated with the Biorad Bradford protein assay [41]. To determine a potential direct effect of STS on dCK activity, we added STS (up to 50 nmol/l) directly to the 10 000 g supernatants of untreated cells and determined the dCK activity. The final reaction mixture contained 10 mmol/l ATP, 5 mmol/l $MgCl_2$, 230 µmol/l ^{3}H-dCyd/dCyd mixture (specific activity; 0.04 Ci/mmol), 180 mmol/l Tris–HCl, 30 µmol/l β-mercaptoethanol and lysate of 9.4×10^4 cells. One mM TdR was added to inhibit TK2 mediated phosphorylation of dCyd [42]. The reaction mixture was incubated at 37°C for 15 and 30 min and terminated by heating at 95°C for 3 min, followed by the subsequent addition of 10 µl 5 mmol/l unlabeled dCyd. The substrate (dCyd) was separated from the phosphorylated product (dCMP) by thin layer chromatography on polyethylene imine cellulose layers with distilled water as the eluent. Radioactivity was estimated in a liquid scintillation counter after the addition of 9 ml Optima Gold (Packard Instrument B.V., Chemical Operations, Groningen, The Netherlands). The dCyd phosphorylating activity in the presence of TdR was considered a dCK activity and the activity found without TdR was considered the combined activity of dCK and TK2. The difference in dCyd phosphorylating activity between with and without TdR was considered a TK2 activity with dCyd as a substrate. Enzyme activities were expressed as nmol product formed per hour per 10^6 cells (nmol/h/10^6 cells).

Western blot for dCK, PKCα, E2F1 and RNR

Western blotting was performed as described earlier [43]. In short, proteins were separated on 12.5% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and probed with the primary antibody. Antibody dilutions of 1 : 1000, 1 : 5000, 1 : 500 and 1 : 100 were used to target PKC, dCK, RNR-M2 or E2F1, respectively. This was followed by incubation with the second antibody conjugated to horseradish peroxidase. Immune complexes were visualized by the enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Uppsala, Sweden) and quantified by scanning on a GS-690 Bio-rad scanner (Bio-Rad, Hercules, California, USA). Levels of expression were reported either relative to the SW1573 cells (set at 1 for PKCα) or relative to purified dCK with a His-tag. These values were used to compare the treated cells with the untreated cells and were expressed relative to the untreated cells.

Transfection

NSCLC cells were seeded in complete growth media at a density of 3×10^6 cells per 75 cm^2 culture flask and were incubated overnight. Before transfection, the cells were washed with a serum-free medium. Lipofectamine 2000 was diluted at 1 : 400 in serum-free media and LNA (2500 nmol/l) was diluted at 1 : 20 in serum-free media. First, the lipofectamine mix was added to the cells and after an incubation step of 7 min at 37°C, the LNA mix was added to the cells (final concentration LNA 25 nmol/l) containing lipofectamine (ratio lipofectamine: LNA 5 : 1). Subsequently, the cells were incubated for 5 h at 37°C. The media were removed and the cells were washed twice with the serum-free media. Finally, 10 ml of the culture medium containing 10% FCS, HEPES and penicillin/streptomycin were added before 43 h of incubation at 37°C with or without gemcitabine. Transfection with non-relevant LNA was used as a control.

Statistical analysis

Differences in IC_{50} values, RG, dCK and TK2 activities were evaluated using the *t*-test for unpaired data. The computer program SPSS (version 7.5, SPSS, Inc., Chicago, Illinois, USA) was used for statistical analysis.

Results

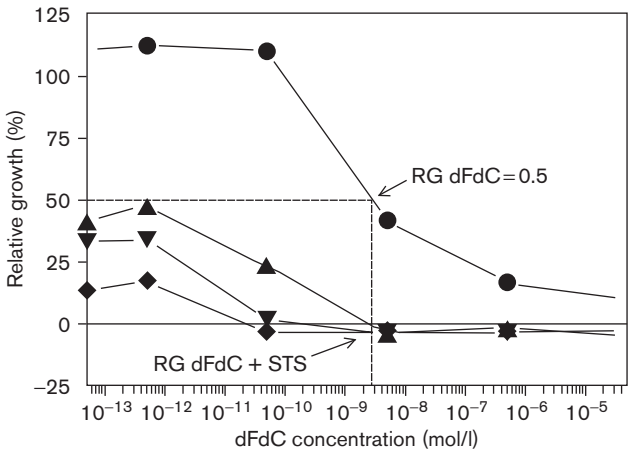
Effect of PKC inhibition by STS on gemcitabine sensitivity

To determine the effect of PKC inhibition on sensitivity to gemcitabine, RG curves were prepared of the cells exposed to gemcitabine and 0, 5, 10 or 50 nmol/l STS for 72 h. Representative growth curves of 2R160 cells exposed to gemcitabine and 0, 5, 10 and 50 nmol/l STS are depicted in Fig 1. The increase in cytotoxicity of the combination of gemcitabine and STS is concentration dependent, resulting in a shift in cytotoxicity to the lower concentration range of gemcitabine at higher concentrations of STS. Moreover, gemcitabine alone did not induce cell kill, but cell kill was found in the cells exposed to gemcitabine in combination with any concentration of STS.

The effect of 5, 10 or 50 nmol/l STS was evaluated by comparison of the cytotoxicity of gemcitabine at its IC_{50} value as a single agent and at this concentration with STS (Fig. 2). STS at these concentrations also caused growth inhibition (Table 1, Fig. 1), which was dependent on the cell line. Five nmol/l STS in combination with gemcitabine did not increase cytotoxicity in SW1573 cells as compared with gemcitabine alone, but in 2R120 and 2R160 cells, 5 nmol/l STS increased cytotoxicity 33-fold and 26-fold ($P = 0.03$), respectively. At 10 nmol/l STS, sensitivity to gemcitabine increased five-fold, 50-fold and 270-fold in SW1573, 2R120 and 2R160 cells, respectively, and at 50 nmol/l STS nine-fold, 1200-fold and 430-fold, respectively. As this evaluation focuses on the effect of gemcitabine cytotoxicity, we also performed synergy analyses.

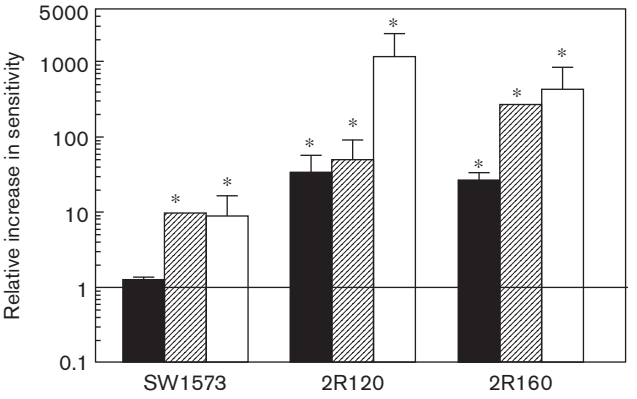
In all the cell lines at all three concentrations of STS, a M/E ratio of less than 1 was found, indicating a synergistic interaction (Table 1). In the SW1573 cells at 5, 10 and 50 nmol/l STS, in 2R120 cells at 5 and 50 nmol/l STS and in 2R160 cells at 50 nmol/l STS, a significant (P value of less than 0.05) difference between the measured and

Fig. 1



Representative growth curves (out of the three separate experiments) of the P-glycoprotein overexpressing non-small cell lung cancer cell line, 2R160, exposed to gemcitabine alone (-●-), gemcitabine and 5 nmol/l staurosporine (STS, -▲-), gemcitabine and 10 nmol/l STS (-▼-) and gemcitabine and 50 nmol/l STS (-◆-) for 72 h. Growth is plotted relative to the not exposed cells. The crossing point of the horizontal dotted line with the gemcitabine curve is used to determine the concentration of gemcitabine causing 50% relative growth (RG). This concentration was selected to calculate an expected growth of the combination and to compare it with the measured growth as shown in the figures. SD was less than 20%. dFdC, 2′,2′-difluorodeoxycytidine

Fig. 2



Effect of 5 nmol/l (■), 10 nmol/l (▨) and 50 nmol/l (□) staurosporine on sensitivity to gemcitabine of the human non-small cell lung cancer cells SW1573 and its multidrug resistance-associated protein and P-glycoprotein overexpressing variants 2R120 and 2R160, respectively (mean ± SEM of 3–5 separate experiments). Cells were cultured for 72 h. The increase in sensitivity was given as the ratio between sensitivity to gemcitabine with staurosporine and of gemcitabine alone. IC_{50} value of gemcitabine in the SW1573 cells was 17 nmol/l, in 2R120 1.9 nmol/l and in 2R160 it was 0.6 nmol/l. *Significantly different from the untreated cells ($P < 0.01$).

Table 1 Evaluation of the interaction between gemcitabine and STS in human NSCLC cell lines

Cell line	Dose STS	Expected relative growth (%)	Measured relative growth (%)	Ratio measured/ expected
SW1573	5	20.8 ± 0.7	17.0 ± 1.0[a]	0.57 ± 0.24
	10	17.5 ± 2.1	2.0 ± 0.0[a]	0.13 ± 0.01
	50	10.7 ± 1.8	0.0 ± 2.5[a]	0.02 ± 0.27
2R120	5	16.7 ± 1.1	−5.3 ± 6.1[a]	<0
	10	14.0 ± 3.7	4.5 ± 3.5	0.41 ± 0.36
	50	13.7 ± 1.6	−0.12 ± 0.0[a]	<0
2R160	5	27.7 ± 2.8	0.18 ± 8.5	0.07 ± 0.04
	10	18.2 ± 1.3	12.5 ± 4.9	0.68 ± 0.27
	50	8.9 ± 1.9	−7.3 ± 2.5[a]	<0

Cells were exposed for 72 h to gemcitabine in a concentration range and STS at fixed concentrations of 5, 10 and 50 nmol/l. Drug interaction was evaluated by calculating ratios between measured relative growth at IC_{50} concentration of gemcitabine alone and expected relative growth (relative growth STS × 0.5). Ratios are mean ± SEM of al least three experiments with this specific approach. A ratio <1 indicates synergism, a ratio >1 antagonism and a ratio of 1 additivity. Relative growth (in %) by exposure to 5, 10 and 50 nmol/l STS was; SW1573: 42.3 ± 1.3, 34.1 ± 3.1, 20.0 ± 2.9%, 2R120: 34.4 ± 1.9, 25.1 ± 5.2, 24.1 ± 3.7%, 2R160: 55.3 ± 5.6, 36.4 ± 1.6, 17.7 ± 3.7%, respectively. A negative ratio represents cell kill of the combination.

NSCLC, non-small cell lung cancer; STS, staurosporine.

[a]Measured value significantly lower than expected value ($P < 0.05$) as determined by a t-test.

expected RG was found. The isobologram analysis confirmed the synergy between gemcitabine and STS (data not shown).

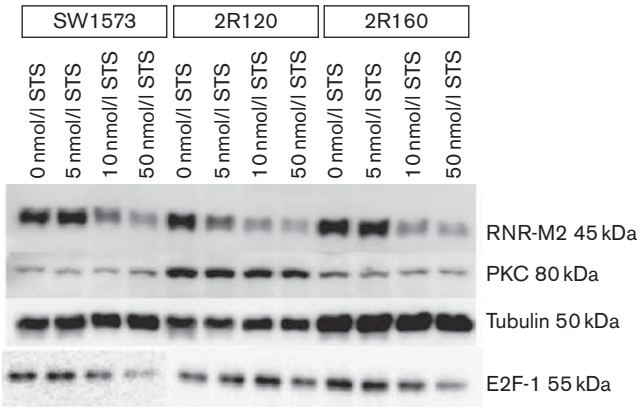
Expression of PKC and PKC regulated proteins

As earlier studies described an increased PKC activity in the cells with a P-gP and MRP overexpression, we determined the expression of the PKC protein in the three cell lines and also studied the effect of STS treatment (Fig. 3). In the MRP overexpressing 2R160 cells PKCα protein expression was only slightly higher than in the SW1573 cells but PKC expression in the P-gP overexpressing 2R120 cells was markedly increased. STS exposure marginally decreased PKC protein expression (Fig. 3). To determine whether STS had an effect on other proteins that could alter gemcitabine sensitivity, we measured the protein expression of RNR. As E2F is associated with the transcription of RNR, we also determined its expression. STS exposure downregulated the protein expression of the catalytic M2 subunit of RNR in increasing concentrations, both in the parental cell line SW1573 and in the sublines R120 and 2R160 (Fig. 3). At a STS concentration higher than 5 nmol/l, the decrease in RNR expression was most clearly observed. Less protein expression of RNR was associated with a downregulation of E2F1 in the SW1573 cells and its subline 2R160 (Fig. 3).

Effect of STS exposure on the dCK and TK2 assays

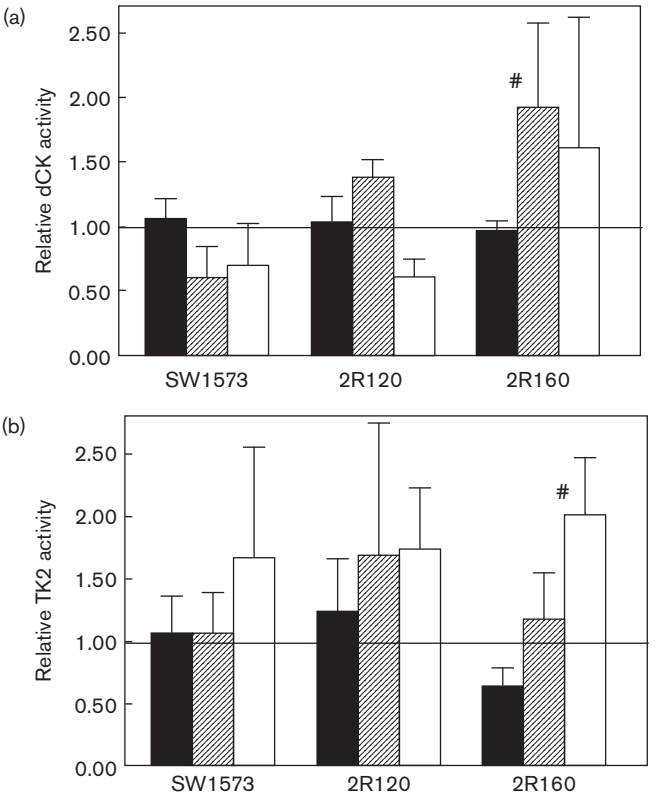
To determine whether alterations in the dCK and TK2 activities induced by a PKC inhibitor would play a role in gemcitabine sensitivity, we treated cells with STS for 24 h (Fig. 4). In the SW1573 cells, no effect on the dCK activity was found, but in the MDR cells 10 and 50 nmol/l STS affected the dCK activity (Fig. 4a); 10 nmol/l STS increased dCK activity 1.4-fold and 1.9-fold in 2R120 and 2R160 cells, respectively. At 50 nmol/l STS, dCK activity decreased in 2R120 but increased in 2R160. The effect on TK2 activity was also concentration dependent in all the cell lines (Fig. 4b), with a marginal effect of 5 nmol/l STS. At 50 nmol/l STS, TK2 activities showed about a two-fold increase in all the cell lines. To determine whether PKC inhibition also affected dCK protein, we estimated the amount of dCK present in the cell by Western blotting (data not shown), which showed that STS did not affect the expression of dCK protein.

Fig. 3



Effect of staurosporine (STS) on protein expression of the M2 subunit of ribonucleotide reductase (RNR), E2F1 and protein kinase C (PKC) in the human non-small cell lung cancer cells SW1573 and its multidrug resistance-associated protein and P-glycoprotein overexpressing variants 2R120 and 2R160 when exposed to 5, 10 and 50 nmol/l STS for 24 h. Loading of the gels was controlled by Tubulin staining.

Fig. 4



Effect of the 24 h treatment with 5 nmol/l (■), 10 nmol/l (▨) and 50 nmol/l (□) staurosporine on deoxycytidine kinase (dCK) (a) and TK2 (b) activities in the human non-small cell lung cancer cells SW1573 and its multidrug resistance-associated protein and P-glycoprotein overexpressing variants 2R120 and 2R160, respectively. dCK activity not exposed cells: SW1573; 0.32 ± 0.05, 2R120; 2.10 ± 0.18, 2R160; 1.29 ± 0.40 in nmol/h/10^6 cells ± SEM and TK2 activity of not exposed cells: SW1573; 0.21 ± 0.10, 2R120; 0.20 ± 0.20, 2R160; 0.09 ± 0.09 in nmol/h/10^6 cells ± SEM (previously published [16]. Values of dCK activity after staurosporine treatment are expressed relative to that of the not exposed cells and are means ± SEM of at least three experiments. #Significance compared with the control at a P value of less than 0.05.

Effect of STS on dCK and TK2 activities in cell free extracts

To determine whether STS would have a direct effect on the enzyme, STS was added to the cell-free extracts

(Fig. 5). In all three cell lines, a concentration dependent pattern on dCK activity was found. In the SW1573 cells, a three-fold increase was observed at 10 nmol/l STS, but at 50 nmol/l and higher no increase was found. In contrast, in the 2R120 and 2R160 cells a plateau of about two-fold increase was observed at 10–50 nmol/l STS. In none of the three cell lines STS affected TK2 activity in the cell-free extracts (data not shown).

Transfection with PKC targeted oligonucleotides

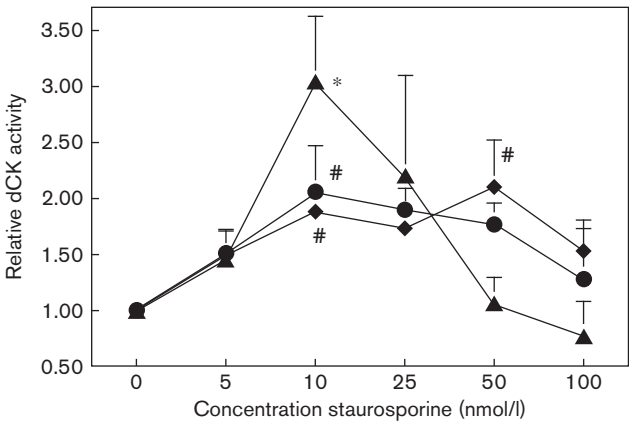
To investigate whether inhibition of PKC-α by STS would affect the dCK activity and hence sensitivity to GEM we transfected SW1573 and three other NSCLC cells with different PKC and dCK expression with LNA oligonucleotides directed to PKC-α. The presence of LNA was followed in time using Cy3-fluorescence attached to the LNA. In the time period of 4–48 h after transfection, the cells were positive (data not shown). PKC protein expression levels were decreased 48 h after transfection in all the cell lines (Fig. 6a); in SW1573 and H292 this was clearly observed at 24 h after transfection. However, when dCK activity was measured at the same time point, we did not observe major changes in the activity of dCK (Fig. 6b); only in the H292 cells we observed a moderate increase in the dCK activity. Therefore, the increase of dCK activity is not because of a direct effect of PKC inhibition. The decrease in PKC did not affect the sensitivity to gemcitabine in any of the cell lines (Fig. 6c).

Discussion

STS increased the toxicity of gemcitabine to the parental NSCLC cells and its MDR variants. This effect was most pronounced in the MDR cells and was associated with an increase in the dCK activity and possibly because of cell cycle modulation, manifested as a decrease in protein expression of E2F1 and the cell cycle regulated RNR M2 subunit.

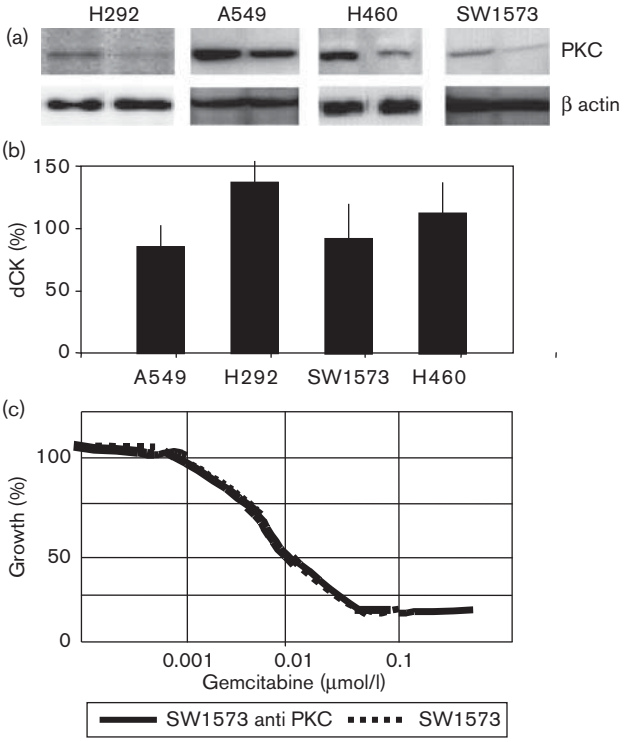
Several reports postulate that inhibition of PKC can mediate an increased sensitivity to deoxynucleoside analogs. The STS analog UCN-01 (7-hydroxystaurosporine) increased cytotoxicity of fludarabine and gemcitabine in several human cell lines, whereas the PKC modulator bryostatin increased that of gemcitabine in human breast cancer cell lines and ara-C in fresh blast cells from patients with AML [26,28]. This effect of bryostatin was accompanied by a downregulation of PKC in gemcitabine treated cells, an increase of the gemcitabine-induced p21 and bax expression and enhanced apoptosis [27]. Bryostatin also increased sensitivity to ara-C, which was related to an increase in the accumulation of the active metabolite ara-CTP [28]. STS also increased ara-C cytotoxicity in HL60 promyelocytic cells [29,44,45], which was associated with an ara-C induced apoptosis in the parental and P-gP and MRP overexpressing variants [29,44]. The more specific PKC inhibitor CGP41251 and

Fig. 5



Effect of staurosporine on deoxycytidine kinase (dCK) activity in the cell-free extracts of the human non-small cell lung cancer cells SW1573 (-▲-) and its multidrug resistance-associated protein and P-glycoprotein overexpressing variants 2R120 (-●-) and 2R160 (-◆-). dCK activities of the cells after staurosporine treatment are expressed relative to the dCK activity of the untreated cells (mean ± SEM) of at least three experiments. Significance compared with the untreated control cells: $^{\#}P<0.05$; $^{*}P<0.02$.

Fig. 6



Transfection with protein kinase C (PKC) α-locked nucleic acid: (a) PKC expression after 48 h in the non-small cell lung cancer cell lines after transfection. (b) Relative deoxycytidine kinase (dCK) activity in the transfected cells. The dCK activity in the locked nucleic acid transfected cells were set on 100%. (c) Growth inhibition after exposure to gemcitabine in the transfected and oligo-transfected SW1573 cells.

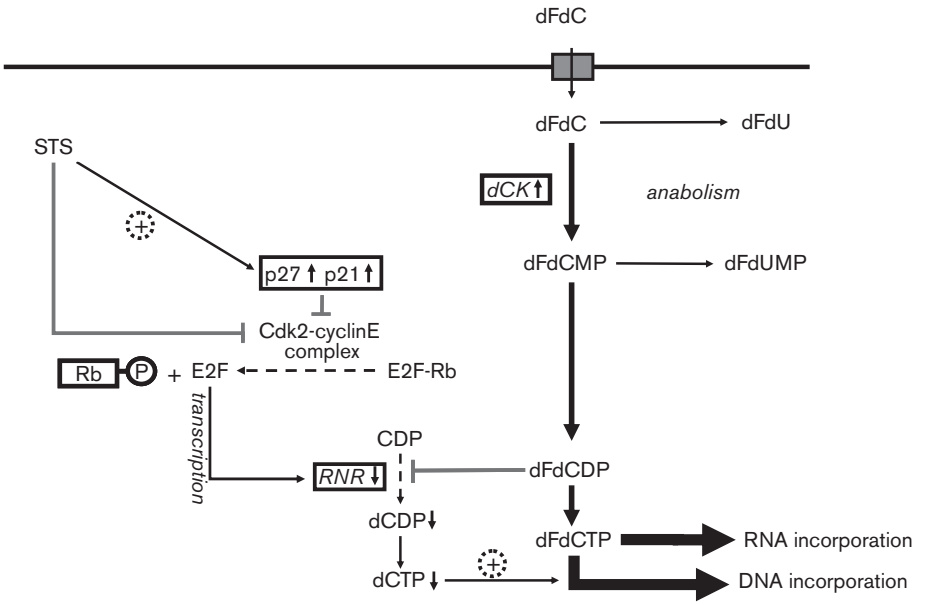
the protein tyrosine kinase (PTK) inhibitor genistein stimulated ara-C induced apoptosis, but to a lesser extent than STS. These data indicated that STS not only inhibited PKC and PTK dependent mechanisms, but also that STS may mediate PKC and PTK independent mechanisms. In all these studies a possible role of PKC inhibition on the activities of gemcitabine metabolizing enzymes was not investigated.

Several studies report an increased PKC expression in MDR cells [17,18], which was indeed found in the MRP overexpressing 2R120 cells, but was minor in the 2R160 cells. In an earlier study we found an increased dCK activity, dCK protein expression and dCK mRNA levels in the 2R120 and 2R160 cells compared with the parental SW1573 cells [16]. This even higher activity was further increased by STS to the same extent in the treated cells and in the cell-free system. This suggests both a direct and indirect effect on dCK as the cell-free system does not include cofactors that are required for the optimal activity of PKC. As exposure to STS did not affect dCK protein levels, regulation of dCK could be at the post-translational level. Wang *et al.* postulated that PKC mediated phosphorylation of dCK [12], which would suggest that inhibition of PKC would decrease the dCK activity. In contrast, PKC was not able to phosphorylate recombinant dCK *ex vivo*, whereas protein kinase A catalyzed dCK phosphorylation [46]. Neither PKC nor protein kinase A increased the activity of dCK with dCyd as a substrate. However, Smal *et al.* [13] provided convincing evidence that dCK can be phosphorylated at the Ser-74 site, but not mediated by PKC. To show/exclude that the effects of STS are mediated by PKC inhibition we downregulated PKC with a specific LNA oligonucleotide. Although PKC was clearly downregulated, no change in the dCK activity was observed, whereas no difference in gemcitabine sensitivity was found; thus, clearly PKC (at least PKC α) does not phosphorylate dCK.

STS also increased the TK2 activity in all the three cell lines. However, in the cell-free assay no direct stimulation of TK2 activity was found, suggesting no direct effect of STS, but that the activity may be regulated by the STS-mediated inhibition of protein phosphorylation. The observed increase was limited to the TK2 located in the mitochondria. For the cytosolic TK1, it has been described that it can be hyperphosphorylated in the M-phase, as the cells arrested in the M-phase were found to have a 10-fold lower affinity for its substrate thymidine than the proliferating cells [47].

STS has different effects on the cell cycle and its regulators (Fig. 7), with a G1 accumulation at low (< 10 nmol/l) concentrations, but a G2 arrest at higher concentrations

Fig. 7



Proposed model for increased gemcitabine sensitivity after staurosporine (STS) exposure. Gemcitabine is transported into the cell by nucleoside transporters and is phosphorylated by deoxycytidine kinase (dCK) [and thymidine kinase (TK) 2] to its monophosphate (dFdCMP) and subsequently to its diphosphate (dFdCDP) and triphosphate (dFdCTP). Gemcitabine triphosphate is incorporated into the RNA and DNA leading to DNA damage. STS leads to increased levels of p21 and p27, which are inactivators of the CDK2-cyclin E complex. Inhibition of CDK2 activity prevents the release of the transcription factor E2F that remains bound the retinoblastoma gene (Rb). Depletion of free E2F prevents transcription of the ribonucleotide reductase (RNR) gene, leading to decreased levels of RNR protein and activity. This will enhance RNR inhibition by dFdCDP leading to the depletion of dCTP. As dCTP is the major feedback inhibitor of dCK, a decrease in dCTP will enhance the dCK-mediated gemcitabine phosphorylation and gemcitabine incorporation into the DNA and increase gemcitabine sensitivity.

[48–50]. A decrease in the levels of CDK2, CDK1, cyclin A and cyclin B proteins was observed upon STS treatment [51], but levels of CDK inhibitors p21 (Waf1/Cip1) and p27 (Kip1) increased [52,53]. These two CDK inhibitors deactivate cyclin E-cdk2 complexes, preventing hyperphosphorylation of Rb and then reduce the release of E2F from the Rb-E2F complex leading to a decrease in RNR. In this study, protein expression of both E2F and the M2 subunit of RNR decreased after STS exposure, which was concentration dependent. Earlier we observed that STS also downregulated another cell cycle dependent protein, thymidylate synthase (TS), leading to an enhanced sensitivity to 5FU, which acts as a TS inhibitor [54]. This is probably the result of a decrease in the CDK2 activity because of the upregulation of p21 and p27, which will impair Rb phosphorylation. Transcription of the S phase related genes including TS and RNR cannot occur when the transcription factor E2F remains bound to the Rb. As RNR decreased more then E2F1, its downregulation seems, at least partly, to be independent of E2F1 and possibly directly related to the other cell cycle proteins.

Gemcitabine diphosphate is able to inhibit RNR that is involved in dNTP synthesis. This property facilitates a selfpotentiating effect of gemcitabine, as decreased RNR activity results in a depletion of dNTPs, including dCTP, which would lead to a stimulated incorporation of gemcitabine into the DNA. Therefore, the downregulation of RNR by STS provides an additional approach to enhance the inhibition of RNR by gemcitabine. In conclusion, STS increased the sensitivity of human NSCLC cell lines to gemcitabine, which was more pronounced in the MDR variants than in the parental cells. This increase in sensitivity might be related to a STS induced increase of dCK activity and a decrease in the free transcription factor E2F and of the RNR protein after STS exposure. This will result in a decrease in the dCK feedback regulation stimulating gemcitabine incorporation into the DNA leading to increased sensitivity.

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