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Steroids affect collateral sensitivity to gemcitabine of multidrug-resistant human lung cancer cells

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Abstract

Gemcitabine is phosphorylated by deoxycytidine kinase and thymidine kinase 2 and during S-phase incorporated into DNA. The steroids cortisol and dexamethasone, which regulate cell proliferation and gene expression, are pumped out of the cell by the membrane efflux pumps P-glycoprotein and multidrug resistance-associated protein (MRP), which are blocked by verapamil. In parental non-small cell lung cancer (NSCLC) cells (SW1573), 5 μM cortisol and 100 nM dexamethasone decreased sensitivity to gemcitabine. However, both cortisol and dexamethasone only decreased sensitivity with verapamil in MRP (2R120) and P-glycoprotein (2R160) overexpressing variants. Cortisol decreased deoxycytidine kinase activity in SW1573 cells and cortisol with verapamil in 2R120 and 2R160 cells. Dexamethasone with verapamil decreased deoxycytidine kinase activity in 2R160. Cortisol decreased thymidine kinase 2 activity in 2R120 and 2R160 cells. Dexamethasone decreased thymidine kinase 2 activity in SW1573, 2R120 and 2R160 cells. In conclusion, since dexamethasone is frequently used to treat side effects of oncolytic therapy, a decrease of sensitivity to gemcitabine by steroids might be clinically relevant.

Keywords: Gemcitabine; Multidrug resistance; Deoxycytidine kinase activity; Thymidine kinase 2 activity; Corticosteroid; NSCLC (non-small cell lung cancer)

1. Introduction

2′,2′-Difluorodeoxycytidine (gemcitabine) is a deoxycytidine analog which is active against non-small cell lung cancer (NSCLC), in patients and in tumor models, both in vitro and in vivo (Hertel et al., 1990; Boven et al., 1993; Abratt et al., 1994). Gemcitabine and deoxycytidine are phosphorylated by deoxycytidine kinase to their monophosphates. Also the mitochondrial enzyme thymidine kinase 2 phosphorylates deoxycytidine and gemcitabine, however, to a lesser extent than deoxycytidine kinase (Bergman et al., 1999; Eriksson et al., 1991). The monophosphates of gemcitabine and deoxycytidine are subsequently phosphorylated to their triphosphates, which are incorporated into DNA, resulting in DNA damage (Huang et al., 1991). Since gemcitabine requires passage through the S-phase in order to be incorporated, this antimetabolite is predominantly active in fast growing tumors (Huang and Plunkett, 1995).

Steroids, like the natural corticosteroid cortisol and the synthetic steroid dexamethasone, inhibit the proliferation of many cell types including lung cancer cell lines (Nakane et al., 1990; Croxtall and Flower, 1992). Although steroids are known to induce discrete changes in gene expression and protein synthesis (Ivarie and O'Farrell, 1978), the molecular mechanisms involved in cell growth control have not been clarified yet. Several studies report a decrease in thymidine kinase activity, most likely the cytosolic thymidine kinase 1, as a result of corticosteroid exposure in rat and chicken (Tesoriere et al., 1989; Herzfeld and Raper, 1980; Naray et al., 1977; Sakata, 1975). Corticosteroids might have a similar effect on deoxycytidine kinase activity.

In patients, side effects of gemcitabine treatment include nausea and vomiting. The adverse effects of treatment of lung carcinoma is prevented by combinations of anti-emetics and dexamethasone (Cleri et al., 1995). How-

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ever, not much is known about interaction of cytostatic therapy and dexamethasone. Since steroids inhibit cell proliferation and gemcitabine is S-phase dependent, theoretically, steroids would decrease sensitivity to gemcitabine.

It is known that some of the steroid hormones such as cortisol, progesterone and aldosterone are substrates for the plasma membrane drug efflux pumps P-glycoprotein and multidrug resistance-associated protein (MRP) (Endicott and Ling, 1989; Grant et al., 1994; Van Kalken et al., 1993; Mulder et al., 1996). This pump function can be blocked by verapamil (Cornwell et al., 1987). In our previous study an increased sensitivity to gemcitabine was found in P-glycoprotein and MRP overexpressing NSCLC cells, which was related to an increased deoxycytidine kinase activity (Bergman et al., 1998). It was hypothesized that a decrease of intracellular cortisol and dexamethasone concentration as a result of P-glycoprotein or MRP activity might result in an increase in gemcitabine phosphorylation and eventually sensitivity.

In this study we determined whether steroids interact with sensitivity to gemcitabine in cells with a MRP and P-glycoprotein overexpression and whether changes in deoxycytidine kinase and thymidine kinase 2 activity are involved.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Flow Laboratories (Irvine, UK) and fetal calf serum from Gibco (New York, USA), trichloroacetic acid, glutamine and gentamicin from Merck (Darmstadt, Germany), trypsin, sulforhodamine B, cortisol and dexamethasone from Sigma (St. Louis, USA). [5-^{3}H]deoxycytidine (21.9 Ci/mmol) was from Moravek, Brea, CA. All other chemicals were of analytical grade and commercially available.

2.2. Cell culture

The in vitro experiments were performed with the human NSCLC cell line SW1573 and its doxorubicin resistant variants 2R120 (overexpressing MRP) and 2R160 (overexpressing P-glycoprotein), which were grown as monolayers in DMEM at 37°C and 5% CO_2, supplemented with 7.5% heat inactivated fetal calf serum, and 250 ng/ml gentamicin (Kuiper et al., 1990; Keizer et al., 1989). Cells were regularly screened for Mycoplasma contamination by using a rapid detection system with a ^{3}H-labelled DNA probe (Gen-Probe, San Diego, CA, USA) and were found to be negative.

2.3. Chemosensitivity testing

The determination of the IC_{50} (the drug concentration causing 50% growth inhibition) in monolayer cell lines, was performed using the sulforhodamine B assay. The assay was performed using the National Cancer Institute (Bethesda, MD, USA) protocol with some small modifications (Skehan et al., 1990; Keepers et al., 1991). Culture conditions were optimized for all cell lines. Exponential growth during culture was ascertained either by daily counting (when grown in flasks) or by a daily sulforhodamine B assay. From these values a doubling time could be calculated using the following formula:

$$b = \frac{\log 2 \cdot c}{\log(y/a)}$$

in which y represents the number of cells at either 24–72 h, a the number of cells at day 0, c number of hours of cell growth and b the doubling time in h. Doubling times for SW1573, 2R120 and 2R160 were 34.6 ± 2.6, 49.7 ± 3.3 and 49.7 ± 4.1 h, respectively. At day 1, the cells were plated in 96-well plates in different densities, depending on their doubling times (SW1573 8,000, 2R120 15,000 and 2R160 15,000 cells/well) in a volume of 100 μl per well. On day 2, gemcitabine, with or without 5 μM cortisol, 100 nM dexamethasone or 25 μM verapamil, were added in a volume of 100 μl, and cells were exposed for 72 h, followed by precipitation of the cells with 50 μl ice-cold 50% w/v trichloroacetic acid, after which the sulforhodamine B assay was performed. The optical density (OD) was measured at 540 nm. Growth inhibition curves were made of the relative to control ODs of every sulforhodamine B assay. The points were connected by straight lines and the IC_{50} values were determined from the interpolated graph (Peters et al., 1993). IC_{50} values of cells exposed to gemcitabine and steroids were expressed relative to IC_{50} values of cells exposed to gemcitabine alone, which were set at 1.

2.4. Enzyme activities

Deoxycytidine kinase and thymidine kinase 2 activity assays were performed as previously described (Bergman et al., 1999). For determination of the effect of corticosteroids on deoxycytidine kinase activity, the cells were cultured in medium containing no corticosteroids, 5 μM cortisol or 100 nM dexamethasone with or without 25 μM verapamil for 24 h, whereafter cells were harvested and washed in phosphate-buffered saline (PBS). Extracts (10,000 g supernatants) were prepared with cold deoxycytidine kinase buffer (0.3 M Tris–HCl, pH 8.0, 50 μM β-mercaptoethanol) and deoxycytidine kinase was assayed at 37°C at 230 μM deoxycytidine (0.04 Ci/mmol) (pH 7.4) with the equivalent of $5.6–9.4 \times 10^4$ cells with or without 1 mM thymidine, to inhibit thymidine kinase 2

mediated phosphorylation of deoxycytidine (Eriksson et al., 1991). The product was quantified as described (Bergman et al., 1999). The deoxycytidine phosphorylating activity in the presence of thymidine was considered deoxycytidine kinase activity and the activity without thymidine as the sum of deoxycytidine kinase and thymidine kinase 2. The difference was considered thymidine kinase 2 activity with deoxycytidine as a substrate. Deoxycytidine kinase and thymidine kinase 2 activities of cells exposed to steroids were expressed relative to deoxycytidine kinase and thymidine kinase 2 activities of cells not exposed, which was set at 1.

2.5. Statistical analysis

Differences in IC_{50} values, deoxycytidine kinase and thymidine kinase 2 activities were evaluated using a *t*-test for unpaired data. The computer program Statistical Program for the Social Sciences (SPSS) (version 7.5, SPSS, Chicago, IL) was used for statistical analysis.

3. Results

3.1. Effect of cortisol, dexamethasone and verapamil on sensitivity to gemcitabine and proliferation rate of SW1573 and its MDR variants

To investigate the effect of cortisol and dexamethasone on sensitivity to gemcitabine, cells were exposed to gemcitabine with or without corticosteroids. Sensitivities of the cells relative to their sensitivities to gemcitabine alone are depicted in Fig. 1. Cortisol (5 μM) decreased sensitivity to gemcitabine 1.8- and 1.5-fold in SW1573 and 2R160 cells, respectively. However, 2R120 cells were two-fold more sensitive to gemcitabine during cortisol exposure. Cortisol did not affect doubling time of either cell line (data not shown). Dexamethasone (100 nM) decreased sensitivity to gemcitabine 23-fold in SW1573 ($P = 0.02$), but only 1.1- and 1.3-fold in 2R120 and 2R160 cells, respectively. Also dexamethasone did not affect the doubling times of the three cell lines.

A possible role for P-glycoprotein and MRP was investigated by addition of 25 μM of the membrane efflux pump inhibitor verapamil to parental and MDR cells. Verapamil did not affect the combination of gemcitabine and cortisol in SW1573 cells. However, verapamil decreased sensitivity to gemcitabine and cortisol 5-fold in 2R120 cells ($P = 0.01$). Verapamil only slightly enhanced the cortisol induced decrease in sensitivity to gemcitabine in 2R160 cells. Verapamil in combination with cortisol did not affect doubling time of SW1573 and 2R120 cells, but decreased doubling time 1.2-fold in 2R160 cells (data not shown). In SW1573 cells verapamil increased sensitivity to gemcitabine and dexamethasone alone. However, in 2R120 and 2R160 cells verapamil enhanced dexamethasone induced decrease of gemcitabine sensitivity 2.6- and 1.5-fold, respectively. Verapamil and dexamethasone did not affect doubling time of SW1573, but decreased doubling times 1.2- and 1.3-fold in 2R120 and 2R160 cells, respectively (data not shown).

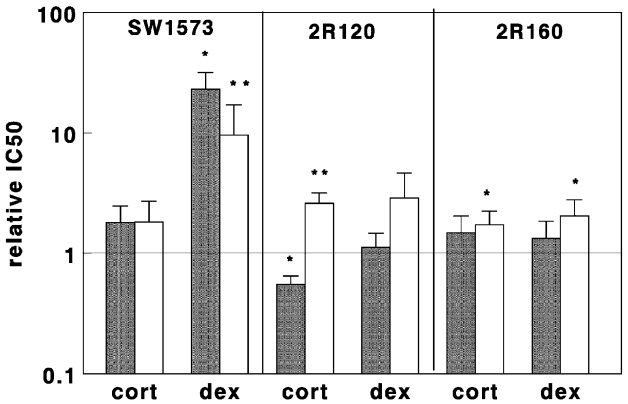

Fig. 1. Effect of cortisol and dexamethasone without (▩) and with (□) verapamil on sensitivity to gemcitabine of the human NSCLC cells SW1573 and its MRP and P-glycoprotein overexpressing variants 2R120 and 2R160, respectively. Cells were exposed to gemcitabine with or without 5 μM cortisol, 100 nM dexamethasone, 5 μM cortisol and 25 μM verapamil or 100 nM dexamethasone and 25 μM verapamil for 72 h. Values are means of sensitivities of the cells to gemcitabine expressed relative to the sensitivity to gemcitabine alone. For each experiment the sensitivity to gemcitabine alone was set at 1; ratio values represent means ± S.E.M. of 3–5 separate experiments. Actual sensitivities; SW1573; 17.00 ± 1.2, 2R120; 1.92 ± 0.68, 2R160; 0.61 ± 0.21 (means ± S.E.M. in nM). * Significantly different from control ($P < 0.05$), ** Significantly different from control and from without verapamil ($P < 0.05$).

3.2. Effect of cortisol, dexamethasone and verapamil on deoxycytidine kinase activity of SW1573 and its MDR variants

Since gemcitabine requires phophorylation in order to be active, we investigated a possible regulation of deoxycytidine kinase and thymidine kinase 2 by corticosteroids. The effects of steroid exposure on deoxycytidine kinase activity are depicted in Fig. 2A. Cortisol (5 μM) slightly decreased deoxycytidine kinase activity in SW1573 cells, but increased activity 1.2-fold in 2R120 and 2R160 cells. Dexamethasone (100 nM) did not affect deoxycytidine kinase activity in SW1573 and 2R160 cells, but increased activity 1.2-fold in 2R120 cells.

The combination of cortisol and 25 μM verapamil resulted in a further decrease in deoxycytidine kinase activity in SW1573 cells. In 2R120 and 2R160 cells the addition of verapamil reverted the 1.2-fold increase in deoxycytidine kinase activity by cortisol alone, in a 1.3- and 1.4-fold decrease in deoxycytidine kinase activity, respectively. However, the differences found between cortisol alone and in combination with verapamil were not significant. Also dexamethasone in combination with verapamil did not alter deoxycytidine kinase activity in SW1573

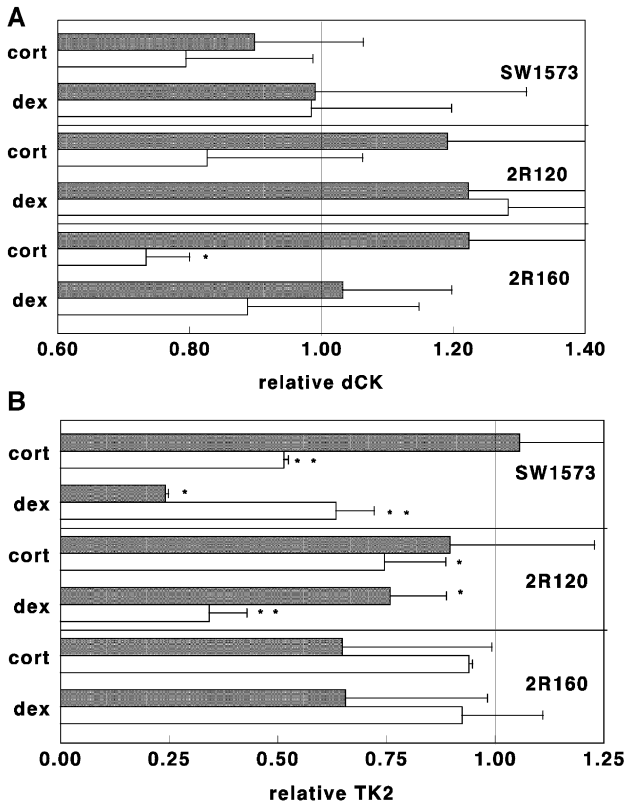

Fig. 2. Effect of cortisol and dexamethasone without (▩) and with (□) verapamil on deoxycytidine kinase (A) and thymidine kinase 2 (B) activity in the human NSCLC cells SW1573 and its MRP and P-glycoprotein overexpressing variants 2R120 and 2R160, respectively. Cells were exposed to gemcitabine with or without 5 μM cortisol, 100 nM dexamethasone, 5 μM cortisol and 25 μM verapamil or 100 nM dexamethasone and 25 μM verapamil for 24 h, whereafter deoxycytidine kinase and thymidine kinase 2 activities were determined. Values are means of deoxycytidine kinase and thymidine kinase 2 activities expressed relative to the deoxycytidine kinase and thymidine kinase 2 activities in not exposed cells, respectively. The enzyme activity in the absence of additives was set at 1; values represent means ± S.E.M. of three to eight separate experiments. Actual deoxycytidine kinase and thymidine kinase 2 activities; SW1573; 0.32 ± 0.05 and 0.21 ± 0.10, 2R120; 2.10 ± 0.18 and 0.20 ± 0.20, 2R160; 1.29 ± 0.40 and 0.09 ± 0.09, respectively (means ± S.E.M. in nmol/h/10^6 cells). * Significantly different from control ($P < 0.05$), ** Significantly different from control and from without verapamil ($P < 0.05$).

cells. In 2R120 cells, verapamil and dexamethasone slightly increased deoxycytidine kinase activity but decreased activity in 2R160 cells.

The effects of steroid exposure on thymidine kinase 2 activity are depicted in Fig. 2B. Cortisol (5 μM) did not affect thymidine kinase 2 activity in SW1573 and 2R120 cells but decreased its activity 1.5-fold in 2R160 cells. Dexamethasone decreased thymidine kinase 2 activity 4.1-, 1.3- and 1.5-fold in SW1573 ($P = 0.02$), 2R120 and 2R160, respectively. Cortisol in combination with verapamil decreased thymidine kinase 2 activity 2- and 1.3-fold in SW1573 and 2R120 cells, respectively, but almost completely reverted cortisol induced decrease in thymidine kinase 2 activity in 2R160 cells. Verapamil partially reverted the decrease in thymidine kinase 2 activity in SW1573 cells and almost completely reverted the decrease in thymidine kinase 2 activity in 2R160 cells. However, verapamil enhanced the dexamethasone induced decrease in 2R120 cells to a 3-fold decrease in thymidine kinase 2 activity ($P = 0.05$).

4. Discussion

In this paper, we describe a decrease in gemcitabine sensitivity by steroid exposure in wild-type NSCLC cells. This is in agreement with a study of Rieger et al. (1999) who described a reduction of gemcitabine sensitivity of cultured human glioma cells by clinically relevant concentrations of dexamethasone. However, in the P-glycoprotein and MRP overexpressing variants no effect was found, which might be mediated by a decrease of steroid concentrations by the action of the membrane efflux pumps. Verapamil reverted this effect in the P-glycoprotein and MRP overexpressing cells, but not in the parental cells. However, in 2R160 cells, only a small decrease in sensitivity to gemcitabine was found by the addition of verapamil, which might be the result of the inhibitory effect of the steroids on P-glycoprotein activity.

Since gemcitabine is incorporated into DNA, sensitivity to this agent is expected to be related to the proliferation rate. Hoffmann et al. (1995) observed that dexamethasone inhibited growth in almost half of the human NSCLC cell lines tested, which was related to high concentrations of glucocorticoid receptors. In this study steroids moderately decreased proliferation rate only in the P-glycoprotein and MRP overexpressing variants in the presence of verapamil. However, the rate of reduction of sensitivity to gemcitabine did not relate to the decrease in proliferation by corticosteroids, suggesting other mechanisms of gemcitabine sensitivity, modulated by steroids.

Cortisol did not affect deoxycytidine kinase activity in the parental cells, but increased deoxycytidine kinase activity in both MDR cells, which was changed to a decrease by verapamil. Since verapamil decreased deoxycytidine kinase activity in the P-glycoprotein overexpressing 2R160 cells, this suggests an important role of the membrane efflux pumps in the control of the intracellular steroid concentration. Cortisol decreased thymidine kinase 2 activity in the P-glycoprotein and MRP overexpressing cells, while the addition of verapamil further decreased thymidine kinase 2 activity in 2R120 cells possibly due to a higher intracellular concentration. The modulation by cortisol and dexamethasone alone (without verapamil) of deoxycytidine kinase and thymidine kinase 2 activity in 2R120 and 2R160 cells, might be explained by a residual concentration of steroids not pumped out of the cell completely. In addition, verapamil decreased thymidine kinase 2 activity further in SW1573 cells. SW1573 cells express some membrane efflux pump activity, although the expres-

sion is much lower compared to the overexpressing 2R120 and 2R160 cells. This might explain the verapamil effect found in SW1573. To our knowledge there are no reports on the effect of cortisol and dexamethasone on deoxycytidine kinase or thymidine kinase 2 activity. However, cytotoxic stress may increase deoxycytidine kinase activity (Spasokoukotskaja et al., 1999). In 2R120 and 2R160 cells, cortisol and dexamethasone may increase deoxycytidine kinase activity by a similar mechanism, but without an increase in sensitivity to gemcitabine. This might be explained by the increased membrane efflux pump activity during steroid and verapamil exposure, requiring ATP. A depletion in ATP pools will result in a UTP depletion, the major phosphate donor for deoxycytidine kinase (Shewach et al., 1995), resulting in lower deoxycytidine kinase activity despite the increased expression of the protein.

Several papers describe an effect of steroids on total thymidine kinase activity, which most likely represents thymidine kinase 1. In contrast to thymidine kinase 2, thymidine kinase 1 does not phosphorylate deoxycytidine and gemcitabine (Eriksson et al., 1991). In suckling rats given injections of cortisol thymidine kinase activity was reduced substantially in several tissues (Herzfeld and Raper, 1980). Also in healthy chickens thymidine kinase activity decreased by 80% after administration of cortisol or dexamethasone (Naray et al., 1977). In P1798 cells thymidine kinase activity decreased with 50% within 8 h exposure to 100 nM dexamethasone (Barbour et al., 1988). The decreased thymidine kinase activity was associated with a reduced abundance of thymidine kinase mRNA, which was attributable to a decrease in the number of RNA polymerase II molecules engaged in transcription of the thymidine kinase gene. With respect to thymidine kinase, there was an overall correlation between enzyme activity, mRNA, and nuclear transcription.

Glucocorticoids also decreased activity of the murine cytosolic enzyme thymidine kinase 1, by inhibition of transcription of the encoding gene (Rhee and Thompson, 1996) and dexamethasone inhibited thymidine kinase 1 activity of murine L cells by 70–90% after 24 h exposure (Frost et al., 1993). This was associated with a 90–95% decrease in thymidine kinase 1 mRNA abundance. The decrease in thymidine kinase 1 mRNA was not caused by a decrease in transcription of the thymidine kinase 1 gene (Frost et al., 1993). Although these authors suggested that regulation of thymidine kinase 1 was entirely due to a posttranscriptional mechanism, most authors suggest that glucocorticosteroids inhibit transcription.

Glucocorticoid receptors have been identified interacting with mitochondrial DNA sequences, suggesting a regulation of gene expression by steroid hormones (Demonacos et al., 1995). Probably thymidine kinase 2 is also regulated by steroid hormones and drugs. Since the substrate specificity of gemcitabine to purified thymidine kinase 2 is 10–20-fold lower than of deoxycytidine (Wang et al., 1999), the role of thymidine kinase 2 in the sensitivity to gemcitabine will be limited. Moreover, thymidine kinase 2 activity is 1.5-, 11- and 14-fold lower than deoxycytidine kinase activity in SW1573, 2R120 and 2R160 cells, respectively (Bergman et al., 1998).

In conclusion, steroids reduce sensitivity to gemcitabine in NSCLC cells. The reduction of gemcitabine sensitivity seems partially due to deoxycytidine kinase and thymidine kinase 2 regulation. P-glycoprotein and MRP activity play an important role in the extent of modulation of sensitivity by steroids. Whether a decrease of sensitivity to gemcitabine by steroids is clinical relevant, requires further research.

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