ELSEVIER

Biochemical Pharmacology 63 (2002) 669–676

Biochemical Pharmacology

# Intracellular localization of 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine in membrane structures impeding the inhibition of cytosolic cyclic AMP-specific phosphodiesterase

Doris Marko[a,*], Karl-Heinz Merz[a], Claudia Kunz[a], Anja Müller[a], Nadya Tarasova[b], Gerhard Eisenbrand[a]

[a]*Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Str. 52, D-67663 Kaiserslautern, Germany*

[b]*Molecular Aspects of Drug Design Section, NCI-Frederick, Frederick, MD, USA*

Received 9 April 2001; accepted 31 October 2001

**Abstract**

7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine (DC-TA-46) is a potent inhibitor of the rolipram-sensitive cAMP-specific phosphodiesterase isoenzyme family PDE4. DC-TA-46 inhibits cAMP-hydrolysis of PDE4 isolated from solid tumors of the human large cell lung tumor xenograft LXFL529 in the nanomolar range ($IC_{50} = 16 \pm 5$ nM). Tumor cells, however, are growth inhibited only in the lower micromolar range as shown for the human large cell lung carcinoma cell line LXFL529L. To investigate reasons for the discrepancy between $IC_{50}$ values for target inhibition and inhibition of cell growth, uptake, subcellular distribution and elimination of the compound were measured. DC-TA-46 was rapidly taken up by the cells, predominantly localized in intracellular membranes. Elimination was slow, with 70% of the compound still persisting in the membranes 50 hr after withdrawal. Confocal laser scanning microscopy showed a clear colocalization with a fluorescent marker for the endoplasmatic reticulum (ER). As a result of the subcellular localization, the membrane-bound PDE activity of LXFL529L cells was effectively inhibited by DC-TA-46 ($IC_{50} = 0.06 \pm 0.02$ μM). In contrast, inhibition of the cytosolic PDE activity was only achieved at concentrations >1 μM ($IC_{50} = 2.0 \pm 0.5$ μM), in the concentration range where also growth inhibition was observed. Thus, the inhibition of the intracellular PDE activity in the different cellular compartments appears to represent an important parameter for the evaluation of the inhibitory properties at least of this class of compounds.


*Keywords:* Phosphodiesterase; cAMP; DC-TA-46; Cellular uptake; Endoplasmatic reticulum; Confocal microscopy

## 1. Introduction

The second messenger cAMP is involved in a multitude of cellular processes including the regulation of cellular proliferation. Many tumor cells have been reported to show significantly decreased intracellular cAMP levels together with increased activity of 3′,5′-cyclic nucleotide phosphodiesterases (PDEs), compared to their nontransformed counterparts. PDEs represent a superfamily of isoenzymes, consisting of at least 11 isoenzyme families differing in substrate specificity, kinetic characteristics and sensitivity to isoenzyme specific modulators [1–8]. The cAMP-specific rolipram-sensitive isoenzyme family PDE4 has been found to represent the highest cAMP-hydrolysing activity in many human tumor cells [9]. DC-TA-46 is a potent PDE4 inhibitor [10,11], inhibiting cAMP hydrolysis of PDE4 isolated from solid tumor tissue of the human large cell lung carcinoma LXFL529 with an $IC_{50}$ of $16 \pm 5$ nM [12]. Treatment of the respective cell line LXFL529L cells with DC-TA-46 induces dose-dependent growth inhibition. Cells are arrested in the $G_1/G_0$ phase of the cell cycle and undergo apoptosis [9]. However, a

* Corresponding author. Tel.: +49-631-205-4061; fax: +49-631-205-3085.

*E-mail address:* marko@rhrk.uni-kl.de (D. Marko).

*Abbreviations:* cAMP, cyclic adenosine monophosphate; ER, endoplasmatic reticulum; FCS, fetal calf serum; IBMX, isobutylmethylxanthine; PDE, phosphodiesterase.

0006-2952/02/$ – see front matter 
PII: S0006-2952(01)00894-2

striking discrepancy is apparent between the potency of DC-TA-46 to inhibit cAMP hydrolysis of isolated PDE4 from solid tumor tissue of LXFL529 and growth inhibitory concentrations in the sulforhodamine B assay for the respective permanent cell line LXFL529L [12]. In the present study, we investigated potential reasons for this difference in effectiveness to elucidate if PDE4 is likely to be the relevant target of the compound for the observed cellular effects like cell cycle arrest and growth inhibition. Therefore, we investigated important parameters of the cellular pharmacokinetics of DC-TA-46 as well as the impact of the compound on the PDE activity in different cellular compartments.

## 2. Materials and methods

### 2.1. Chemicals

DC-TA-46 was synthesized as described previously [12]. Rolipram was kindly provided by Schering AG. Hexyl ester of rhodamine B and Lysotracker Red DND-99 were purchased from Molecular Probes Inc.

### 2.2. Cell culture

The large cell lung tumor xenograft cell line LXFL529L [13] was cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in humidified incubators (37°, 5% $CO_2$). Cell culture medium and supplements were obtained from Gibco Life Technologies. Cells were routinely tested for the absence of mycoplasm contamination.

### 2.3. Sulforhodamine B assay

Effects on cell growth were determined as described previously [14]. Briefly, cells were seeded into 24-well plates (4000 cells per well) and allowed to grow for 24 hr before treatment. Thereafter, cells were incubated for 24 hr with DC-TA-46 in the presence or absence of serum. Incubation was stopped by addition of trichloroacetic acid (50% solution). After 1 hr at 4°, plates were washed four times with water. The dried plates were stained with a 0.4% solution of sulforhodamine B. The dye was eluted and quantified photometrically at 570 nm. Cytotoxicity was determined as percent survival, determined by the number of treated over control, cells × 100 (i.e., *%T/C*).

### 2.4. Cellular uptake and elimination

LXFL529L cells, seeded into 10-cm petri dishes, were cultivated until approximately 70% confluence. Medium was removed, cells were washed with PBS (5 mL) and subsequently incubated with 1 μM DC-TA-46 in serum-containing medium. For determination of the elimination of the compound, cells were incubated with drug containing medium for 24 hr. Thereafter, medium was removed, the monolayer was washed with PBS (5 mL), and cell culture was continued using drug-free medium.

At the end of the respective incubation time, cells were washed twice with PBS (5 mL), harvested by scraping into PBS (2 × 500 μL) and cracked by three cycles of freezing and thawing in liquid nitrogen. For each incubation time three petri dishes were treated in parallel and pooled after cell harvest. The protein content of the samples was determined using the BCA assay (Pierce Chemical Company). Separation of cytosolic and particulate fraction was achieved by centrifugation at 100,000 *g* for 1 hr. The pellet was resuspended in 0.5 N NaOH (1.00 mL). The solution was stirred at room temperature for 4 hr and acidified with 0.5 N HCl (1.05 mL).

Enrichment and purification of the drug was provided by solid phase extraction (SPE column: LiChrolut RP-18e, 200 mg; Merck). The methanolic eluate was evaporated to dryness. A defined volume of methanol was added and this solution was applied to HPLC. Precolumn: LiChroCART 4-4 with LiChrospher 100 RP-18e, column: LiChroCART 125-4 with LiChrospher RP-18e 100, 5 μm (Merck), eluent: 65% 0.3 M $NaH_2PO_4$, 35% acetonitril, adjusted to pH 5.0 by 70% $HClO_4$; flow 1 mL/min at room temperature, UV-detection at 282 nm. Each sample was analyzed at least 3-fold. Quantification was performed by comparison with an external standard. Limit of detection was about 0.5 μM.

### 2.5. Protein binding

BSA (40 mg/mL in PBS) was incubated with 1 μM DC-TA-46 for 15 min at 37°. Subsequently, the solution was centrifuged at 4000 *g* for 45 min Centrikon® microconcentrators (Amicon GmbH) with an exclusion size of 30,000 Da (membrane YM3) according to the manufacturers instructions. Drug concentration in the filtrate and the retentate was determined by SPE, HPLC/UV as described above.

### 2.6. Phosphodiesterase activity

Solid tumor tissue of the human xenograft LXFL529 was prepared as described previously [12]. For experiments with the respective cell line LXFL529L, cells were seeded in 10-cm petri dishes and treatment started at a cell density of 60–70% (logarithmical growth). For each experiment three petri dishes were treated in parallel. Cells were incubated with DC-TA-46 for 24 hr in the presence of serum. Before harvesting, medium was removed and cells were washed with 5 mL of the homogenization buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 4 mM benzamidine hydrochloride, 0.5 μM trypsin inhibitor from soy beans, 0.1 mM phenylmethylsulfonylfluoride, 1 mM β-mercaptoethanol, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 1 μM pepstatin,

1 μM leupeptin). Harvesting and lysate preparation was performed at 4°. Cells were harvested by scraping in homogenization buffer (2 × 500 μL) and were lysed by three cycles of freezing and thawing in liquid nitrogen. After centrifugation (10,000 *g*, 15 min), the supernatant (soluble fraction) was carefully removed and directly subjected to the PDE assay. The remaining pellet was treated with Tris–buffer containing 0.5% Triton® X-100 for solubilization of the membrane proteins. After centrifugation (10,000 *g*, 15 min), the supernatant (solubilized membrane fraction) was removed and subjected to the PDE assay. Untreated cells were cultivated and harvested in parallel. After the preparation of the soluble and particulate fraction, PDE activity was determined in the presence of DC-TA-46. Protein content of all samples was determined according to the method of Bradford.

PDE activity was determined as described previously [10,11]. Briefly, PDE-containing samples were incubated at 37° with a mixture of cAMP and [$^3$H]-cAMP in a buffer containing 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM AMP, resulting in a final cAMP concentration in the assay of 1 μM. Reaction was stopped at a maximal cAMP turnover of 20% by adding $ZnSO_4$. [$^3$H]-5′-AMP was precipitated by the addition of $Ba(OH)_2$ and centrifugation at 10,000 *g* for 5 min. Nonhydrolysed [$^3$H]-cAMP was determined by liquid scintillation counting of the supernatant. PDE activity of each sample was determined in triplicate. The whole experiment was performed three times.

### 2.7. Confocal laser scanning microscopy

LXFL529L cells were grown in Nunc chamber slides with removable walls in medium without phenol red and were incubated with 2 μM DC-TA-46 at 37° for various length of time. LysoTracker Red was applied at 10,000-fold dilution for 15 min and ER marker hexyl ester of rhodamine B was applied at 1 μM concentration for 20 min. After incubation with the compound and organelle markers, the cells were rinsed with PBS, the walls of the slides were removed and the cells were covered with glass cover slips. The images of the cells were taken on Zeiss 310 confocal laser scanning microscope using 40× water lens. Fluorescence of DC-TA-46 was excited with 364-nm ion laser, and emission was detected using a 450–490 nm filter. For LysoTracker Red and hexyl ester of rhodamine B, a 543 helium/neon laser was used for excitation and fluorescence was detected with a 590-nm cut-off filter. Electronic zoom 5X was used for all images.

## 3. Results

### 3.1. Impact of protein binding

Growth inhibition studies with DC-TA-46 were performed by incubation of cultured cells for 72 hr in the presence of serum [12]. Incubation of 1 μM DC-TA-46 with BSA revealed a high protein binding affinity, 89 ± 2% of the drug being bound to the protein. To test the impact of this high protein binding on the growth inhibitory properties of DC-TA-46, LXFL529L cells were treated for 24 hr with the compound in the presence or absence of FCS (Fig. 1). In the absence of serum an $IC_{50}$ value of 5.2 ± 0.5 μM was determined in the sulforhodamine B assay. Only a slight increase of the $IC_{50}$ value to 8.0 ± 0.4 μM was observed in the presence of 10% FCS. Thus, despite the high protein binding properties of the compound, growth inhibition is only marginally affected by incubation in serum containing medium.

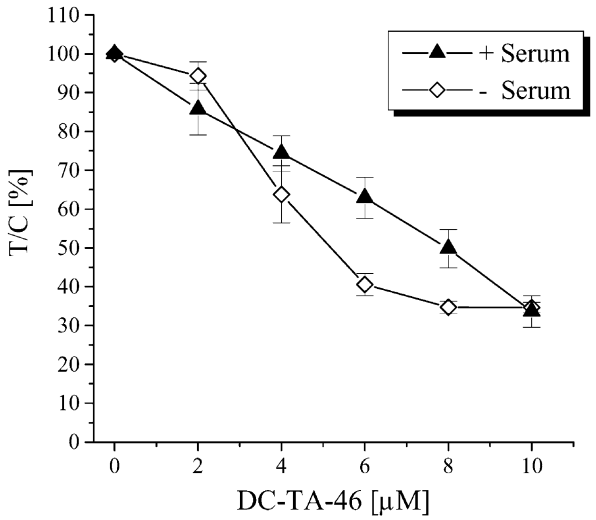


Fig. 1. Effect of serum on the growth inhibitory properties of DC-TA-46 on LXFL529L cells. Cells were incubated with the compound for 24 hr in the presence or absence of serum: (▲) incubation in the presence of FCS; (◇) in the absence of serum. Growth inhibition was determined using the sulforhodamine B assay [14]. Data are calculated as percent compared to the vehicle control (*% T/C*). Values are given as the mean ± SD of three separate experiments, each done in quadruplicate.

### 3.2. Cellular uptake and elimination

Using fluorescence microscopy, it was observed that DC-TA-46 was rapidly taken up by LXFL529L cells, but was not evenly distributed. After incubation with 1 μM DC-TA-46 for 30 min, the compound was nearly exclusively located in the perinuclear region of the cells (data not shown). The invasion of DC-TA-46 into LXFL529L cells was determined using HPLC with UV detection (Fig. 2). The time course of invasion for both, cytosolic and particulate fraction can be described by the equation

$$M_{inv} = M_{max}(1 - e^{-k_{inv}t}) \quad (1)$$

where $M_{inv}$ is the amount of drug in the respective compartment at time *t*. $M_{max}$ is the amount of DC-TA-46 in the respective compartment at steady state, and $k_{inv}$ is the velocity constant of the invasion. The time of half maximal drug concentration $t_{1/2}$, with $M_{inv} = 0.5M_{max}$, is given by $t_{1/2} = \ln 2/k_{inv}$. Determination of $k_{inv}$ and $t_{1/2}$, respectively,

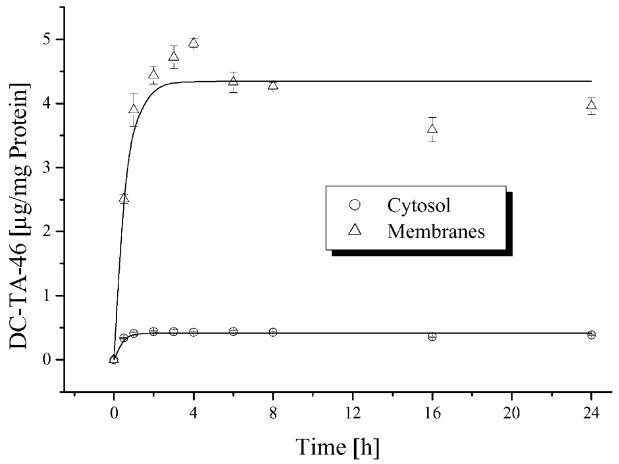


Fig. 2. Invasion of DC-TA-46 into LXFL529L cells (1 μM) analyzed by HPLC with UV detection. Cytosol and particulate were separated by centrifugation at 100,000 *g*. Values are given as the mean ± SD of three separate experiments, each done in triplicate. The fitting function was determined by non-linear curve fitting (software: Origin, MicroCal Software Inc.), parameters see Table 1.

is achieved by nonlinear curve fitting (software: origin, MicroCal Software Inc.) of function (1) to the mean values. The parameters of the fitting function are summarized in Table 1. The cellular uptake of DC-TA-46 proceeded rapidly. By incubation with 1 μM DC-TA-46, half maximal drug concentration was reached already after 12 min in the cytosol and after 20 min in the membranes. At steady state, more than 90% of the drug was localized in the particulate fraction (Fig. 2).

After 24 hr incubation with DC-TA-46, the drug-containing medium was removed and the cellular contents were determined using solid phase extraction followed by HPLC with UV detection. The elimination from the membrane compartment proceeded slowly, with about 80% remaining 24 hr after withdrawal of the compound (Fig. 3). Even 50 hr after withdrawal, more than 70% of the compound was still persisting in the particulate fraction. In contrast, more than 50% of the compound was eliminated from the cytosolic compartment during the first 24 hr after withdrawal. Thereafter, elimination slowed down drastically, with about 40% of the compound still present 50 hr after withdrawal (Fig. 3).

Table 1
Parameter of the fitting function (1) for the invasion of DC-TA-46 (1 μM, 24 hr incubation) into cytosol and membranes of LXFL529L cells calculated by nonlinear curve fitting (Origin, MicroCal Software Inc.)

| Parameter | Cytosol | Particulate |
|---|---|---|
| $M_{max}$ (μg/mg protein) | 0.42 ± 0.01 | 4.34 ± 0.16 |
| $k_{inv}$ ($hr^{-1}$) | 3.40 ± 0.73 | 1.95 ± 0.44 |
| $t_{1/2}$ (hr) | 0.20 ± 0.06 | 0.35 ± 0.12 |
| Correlation coefficient | 0.98 | 0.96 |

### 3.3. Subcellular localization

Confocal laser scanning microscopy of LXFL529L cells treated for 1 hr with DC-TA-46 (2 μM) showed a predominant localization of the fluorescent drug in distinct structures in the perinuclear region of the cells (Figs. 4A and 5A). However, no fluorescence was observed in the nucleus, as seen by comparison with the respective Nomarski image (Figs. 4D and 5D). In living cells, LysoTracker Red® is a fluorescent marker for acidic organelles such as lysosomes, which were readily detectable in LXFL529L cells (Fig. 4B, red). The overlay of the

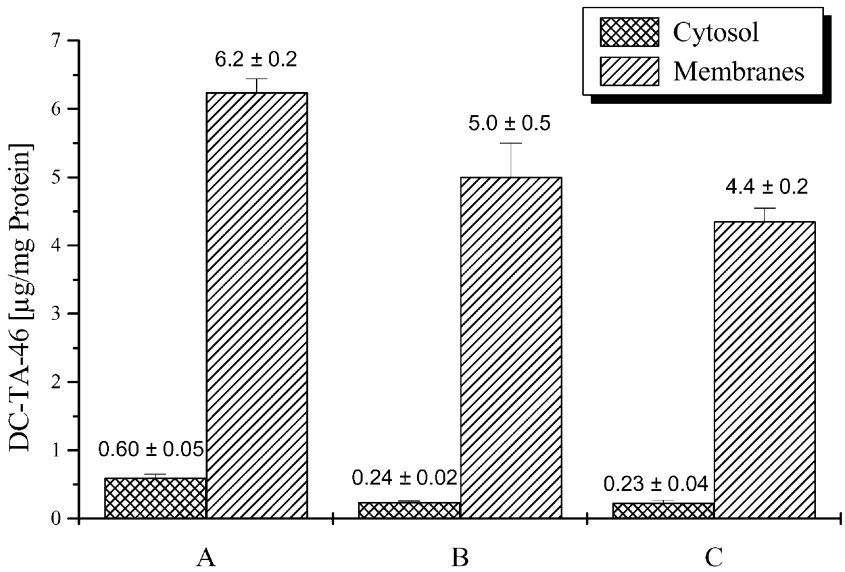


Fig. 3. Content of DC-TA-46 in cytosol and particulate of LXFL529L cells. (A) After 24 hr incubation with 1 μM DC-TA-46; (B) 24 hr after withdrawal of the compound; (C) 50 hr after withdrawal of the compound. Values are given as the mean ± SD of three independent experiments, each performed in triplicate.

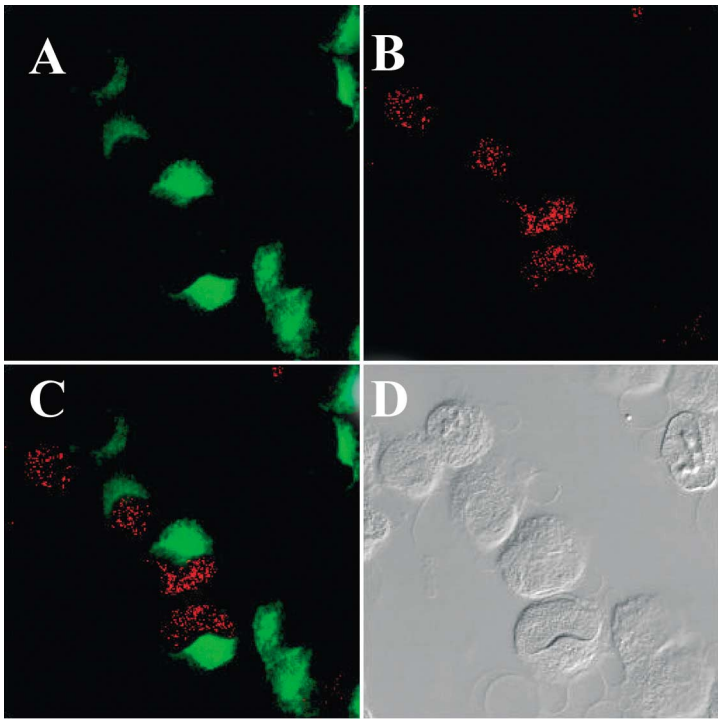


Fig. 4. Confocal laser scanning microscopy image of LXFL529L cells treated for 1 hr with 2 μM DC-TA-46 and subsequently for 15 min with LysoTracker Red® for staining of lysosomes. (A) Green fluorescence of DC-TA-46; (B) Red fluorescence of LysoTracker Red®; (C) Overlay of A and B, colocalization of DC-TA-46 and LysoTracker Red® would appear in yellow; (D) Nomarski image.

fluorescent signals of DC-TA-46 and the lysosomal marker showed clearly a different localization pattern (Fig. 4C).

The hexyl ester of rhodamine B represents a fluorescent marker for the ER. In LXFL529L cells staining of the respective structures was readily detectable (Fig. 5B). An overlay of the fluorescent signals of DC-TA-46 (Fig. 5A) and the hexyl ester of rhodamine B (Fig. 5B) showed a nearly complete colocalization (Fig. 5C).

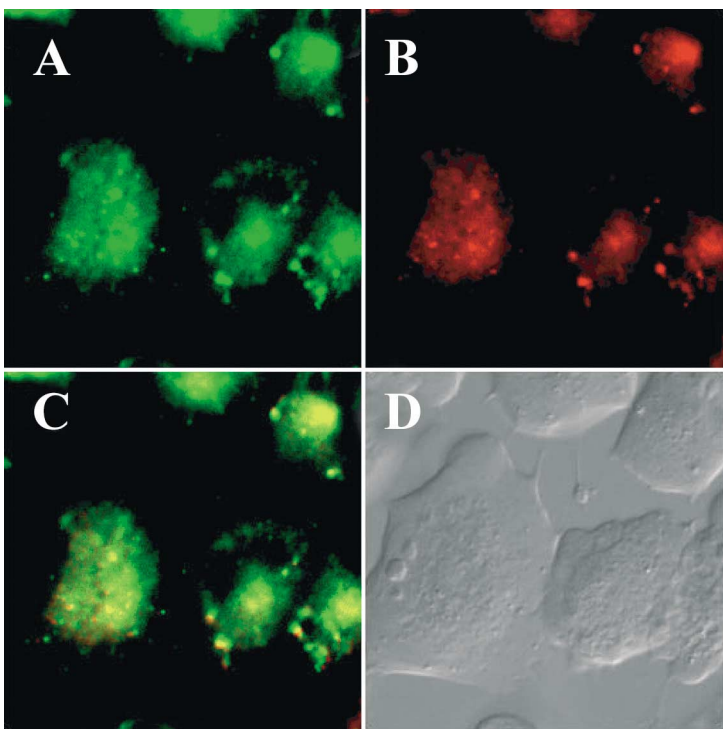


Fig. 5. Confocal laser scanning microscopy image of LXFL529L cells treated for 1 hr with 2 μM DC-TA-46 and subsequently for 20 min with 1 μM of the hexyl ester of rhodamine B for staining of the ER. (A) Green fluorescence of DC-TA-46; (B) Red fluorescence of the hexyl ester of rhodamine B; (C) Overlay of A and B, colocalization of DC-TA-46 and the hexyl ester of rhodamine B appears in yellow; (D) Nomarski image.

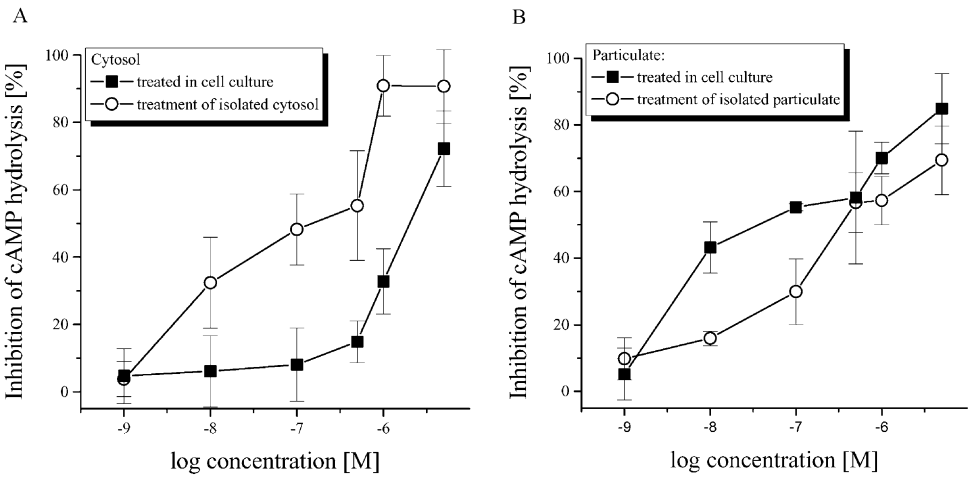


Fig. 6. Inhibition of cytosolic and particulate PDE activity of LXFL529L cells by DC-TA-46. (○) LXFL529L cells were cultivated in the absence of the test compound. After harvesting, cytosol and particulate were separated. The assay for PDE activity was performed in the presence of the respective concentrations of DC-TA-46. (■) LXFL529L cells were treated for 24 hr with the respective drug concentrations. After harvesting, cytosol and particulate were separated and assayed for PDE activity. Cytosolic PDE activity in the untreated control: $122.5 \pm 44.2$ (pmol/min mg protein); particulate PDE activity in the control: $16.9 \pm 7.3$ (pmol/min mg protein). For each assay, two petri dishes were treated in parallel and assayed independently. The PDE assay was performed in triplicate. The whole experiment was repeated three times, values are given as mean ± SD The $IC_{50}$ values are determined as the mean of the $IC_{50}$ values of the different experiments, each calculated by linear regression.

### 3.4. Inhibition of intracellular PDE activity

Furthermore, we investigated the impact of the intracellular localization of the compound on the inhibition of PDE activity in the different cellular compartments. First, we determined the effect of the compound on PDE activity of separate preparations of cytosol and cellular membranes. Untreated cells were harvested, cracked in liquid nitrogen and centrifuged for 15 min at 10,000 *g*. The supernatant (soluble fraction) was carefully removed and directly applied to the PDE assay. PDE activity in the particulate fraction was determined after solubilisation using Triton® X-100. DC-TA-46 was directly added to the respective protein preparations in the PDE assay.

To investigate the effect of DC-TA-46 on the intracellular PDE activity in intact cells, LXFL529L cells were treated for 24 hr with the compound. Thereafter, the cells were harvested and prepared to obtain separate cytosolic and particulate fraction as described above. The cAMP-hydrolysing activity was determined in the PDE assay. We found a significant difference between the potency of DC-TA-46 to inhibit cytosolic PDE activity, when added directly to cytosolic preparations in the PDE assay as compared to the intracellular inhibition of cytosolic PDE achieved after 24 hr treatment of cells with the compound (Fig. 6A). When DC-TA-46 was added directly to cytosolic preparations in the assay, already at 10 nM concentration a significant inhibition of cAMP-hydrolysis was observed ($IC_{50} = 0.22 \pm 0.09$ μM). However, when applied to intact cells, significant inhibition of the cytosolic PDE activity was achieved only at concentrations $\geq 1$ μM ($IC_{50}$ of $2.0 \pm 0.5$ μM, Fig. 6A).

In the particulate fraction, a completely different picture emerged. When added directly to the particulate fraction DC-TA-46 was clearly less active ($IC_{50} = 0.5 \pm 0.25$ μM) as compared to the cytosolic preparation. In intact cells, however, already at 10 nM DC-TA-46 a significant inhibition of particulate PDE activity was achieved ($IC_{50} = 0.06 \pm 0.02$, Fig. 6B). Thus, in the particulate fraction of cells treated for 24 hr with the compound, a significantly stronger inhibition of PDE activity was observed compared to the effects achieved by adding DC-TA-46 directly to particulate preparations in the PDE assay.

## 4. Discussion

The major purpose of the present study was to elucidate potential causes for the discrepancy in effectiveness of the pteridine derivative DC-TA-46. Although this compound inhibits isolated PDE4 in the nanomolar range, cell growth is blocked only at micromolar concentrations. The present investigation addressed the question whether PDE4 is likely a relevant target for the cellular effects of that compound, such as cell cycle arrest and apoptosis induction [9,11].

Rolipram-sensitive PDE4 isoenzymes represent the highest cAMP hydrolyzing activity in solid tumor tissue of the human large cell lung tumor xenograft LXFL529 grown onto nude mice, as well as in the respective cell line [9]. In both, solid tumors and cultured cells of LXFL529, more than 85% of the total PDE activity was localized in the cytosol. Isolated cytosol from the cell line and from tumor tissue as well as respective membrane preparations showed comparable sensitivity to the PDE inhibitory properties of DC-TA-46. The expression pattern of PDE4 subtypes as analyzed by RT-PCR, did not show significant

differences between solid tumor tissue and cell line in the expression of PDE4 subtypes on the mRNA level [9]. Furthermore, DC-TA-46 does not discriminate between different PDE4 subtypes (data not shown). Taken together, these results argue against differential PDE expression to be responsible for the discrepancy in concentration between inhibition of PDE4 isolated from solid tumor tissue and growth inhibition of the respective cell line. However, it can not totally be excluded that during extraction procedures modifications of the protein occur which might affect the sensitivity of the enzyme towards DC-TA-46.

We also addressed the question whether the presence of serum in the cell culture medium influences the growth inhibitory potential of DC-TA-46. High serum binding of compounds might impede drug uptake, thus diminishing potential growth inhibitory effects. However, despite the high protein binding properties of the compound, growth inhibition is only marginally affected by incubation in serum-containing medium (Fig. 1). The resulting difference is not sufficient to explain the gap between PDE4 inhibition and growth inhibitory properties.

Drug uptake and subcellular distribution in LXFL529L cells was studied by HPLC analysis using UV detection (Fig. 2). The compound was rapidly taken up into cells, however, a significantly higher proportion of the compound was detected in the particulate fraction, as compared to the cytosol. In steady state, less than 10% of the compound were detected in the cytosolic compartment. This is in accordance with the results from fluorescence microscopy. As a lipophilic compound, DC-TA-46 is taken up rapidly, migrating to cellular membrane compartments in the perinuclear region of the cell (data not shown). As shown by confocal laser scanning microscopy (Fig. 5) the compound co-localized with the hexyl ester of rhodamine B, a marker for the ER (Fig. 4). Binding to this compartment appears to be rather strong, as to be seen by the slow evasion of the drug after withdrawal (Fig. 3).

In contrast to the predominant localization of DC-TA-46 in cellular membranes, the target enzyme PDE4, however, is predominantly (>85%) cytosolic [9]. The different subcellular localization of the target enzyme and the drug is highly relevant to PDE inhibition by DC-TA-46 in the different cellular compartments (Fig. 6A and B). DC-TA-46 is highly effective on cytosolic PDE activity, when directly applied to cytosolic preparation. In intact cells, however, only at concentrations $\geq 1$ μM significant inhibition of cytosolic PDE activity is achieved (Fig. 6A). This is a concentration range known to also inhibit cell growth [12].

As a consequence of predominant localization and persistence of DC-TA-46 in cellular membranes of intact cells PDE inhibition in that compartment is much stronger than to be expected when applying DC-TA-46 directly to particulate preparations (Fig. 6B). The potent PDE inhibition observed in the membrane compartment of cells clearly shows also that differences between results from cells in culture and those from cellular preparations are not merely a reflection of dilution by cell harvesting. The results further indicate that the effective inhibition of the cytosolic PDE activity is important for the growth inhibitory effects. However, as shown in the accompanying paper, PDE inhibition in the cytosol alone is not sufficient for effective growth inhibition. Enhancement of the intracellular cAMP-level without simultaneously blocking membrane-bound PDEs, results in a rapid upregulation of cAMP-hydrolyzing activity in that compartment, as shown for the combination of the adenylate cyclase stimulator forskolin and the unspecific PDE inhibitor IBMX [15]. Forskolin is known to effectively enhance the intracellular cAMP-levels. However, in LXFL529L cells, forskolin alone or in combination with IBMX showed only marginal growth inhibitory properties (data not shown). The potential growth inhibitory effect of the cAMP increase appears to be compensated by the rapid induction of PDE activity in cellular membranes. This suggests that blocking of PDE activity both, in cytosol *and* membranes, is required for effective growth inhibition.

On the basis of these and earlier results published elsewhere [9–12] it is reasonable to assume that the inhibition of PDE4 in human tumor cells at least contributes substantially to the growth inhibitory properties of DC-TA-46. However, due to its subcellular localization, the inhibition of the cytosolic PDE activity appears to be limiting for its growth inhibitory potency. Although blocking of cytosolic PDE activity appears to be crucial, inhibition of PDE activity in the membrane compartment is also required for effective growth inhibition. Therefore, when aiming at further improvement of PDE4 inhibitors with respect to tumor cell growth inhibitory properties, screening for enzyme inhibition using isolated preparations or recombinant protein might not be sufficient and should be supplemented by assessing the effects on the PDE activity in the different compartments of intact cells.

## Acknowledgments

We thank Prof. Dr. H.H. Fiebig, Freiburg (Germany) for generously providing us solid tumor tissue of the human large cell lung tumor xenograft LXFL529. This work was supported by the Deutsche Forschungsgemeinschaft Grant Ei 172/5-1/2.

## References

[1] Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol Sci 1990;11(4):150–5.

[2] Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB. Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. J Biol Chem 1998;273(25):15559–64.

[3] Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB. Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. Biochem Biophys Res Commun 1998;246(3): 570–7.

[4] Fujishige K, Kotera J, Michibata H, Yuasa K, Takebayashi S, Okumura K, Omori K. Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). J Biol Chem 1999;274(26):18438–45.

[5] Soderling SH, Bayuga SJ, Beavo JA. Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. J Biol Chem 1998;273(25):15553–8.

[6] Yuasa K, Kotera J, Fujishige K, Michibata H, Sasaki T, Omori K. Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue-specific expression. J Biol Chem 2000;275(40):31469–79.

[7] Kotera J, Fujishige K, Yuasa K, Omori K. Characterization and phosphorylation of PDE10A2, a novel alternative splice variant of human phosphodiesterase that hydrolyzes cAMP and cGMP. Biochem Biophys Res Commun 1999;261(3):551–7.

[8] Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA, Phillips SC. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc Natl Acad Sci USA 2000;97(7):3702–7.

[9] Marko D, Pahlke G, Merz KH, Eisenbrand G. Cyclic 3′,5′-nucleotide phosphodiesterases: potential targets for anticancer therapy. Chem Res Toxicol 2000;13(10):944–8.

[10] Drees M, Zimmermann R, Eisenbrand G. 3′,5′-Cyclic nucleotide phosphodiesterase in tumor cells as potential target for tumor growth inhibition. Cancer Res 1993;53(13):3058–61.

[11] Marko D, Romanakis K, Zankl H, Fuerstenberger G, Steinbauer B, Eisenbrand G. Induction of apoptosis by an inhibitor of cAMP-specific PDE in malignant murine carcinoma cells overexpressing PDE activity in comparison to their nonmalignant counterparts. Cell Biochem Biophys 1998;28(2–3):75–101.

[12] Merz KH, Marko D, Regiert T, Reiss G, Frank W, Eisenbrand G. Synthesis of 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine and novel derivatives free of positional isomers. Potent inhibitors of cAMP-specific phosphodiesterase and of malignant tumor cell growth. J Med Chem 1998;41(24):4733–43.

[13] Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR, Combined in vitro/in vivo test procedure with human tumor xenografts for new drug development. In: Fiebig HH, Berger DP, editors. Contributions to oncology. Vol. 42: Immunodeficient mice in oncology. p. 321–51. Karger: Basel; 1992.

[14] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.

[15] Wagner B, Jakobs S, Eisenbrand G, Marko D, Jakobs S, Habermayer M, Hippe F, Cho-Chung YS. 7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine, a potent inhibitor of cAMP-specific phosphodiesterase, enhancing nuclear protein binding to the CRE consensus sequence in human tumor cells. Biochem Pharmacol 2002;63:659–68.