Clodronate inhibits adrenocortical cell proliferation and P450c21 activity

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Abstract

Bisphosphonates (BPs), specific inhibitors of osteoclasts, are well established in the management of skeletal metastases in breast cancer. Recent studies have suggested that these compounds may also directly influence tumor cell proliferation. As adrenocortical cancer frequently leads to bone metastases, we investigated the effects of clodronate (CLO) in the human adrenocortical cancer cell line NCI-H295 and in primary cultures of bovine adrenocortical cells. Both the non-amino BP CLO and the amino BP pamidronate (PAM) exhibited a dose-dependent antiproliferative effect in both cell types (cell viability in NCI-H295 cells: 100 μ M CLO: $98 \pm 8\%$; 500 μ M CLO $76 \pm 6\%$; 1000 μ M CLO $53 \pm 2\%$; 10 μ M PAM $96 \pm 3\%$; 50 μ M PAM $46 \pm 6\%$; 100 μ M PAM $11 \pm 1\%$ vs untreated control cells: $100 \pm 10\%$; $P < 0.01$). FACS analysis in NCI-H295 cells treated with either CLO or PAM demonstrated both apoptotic and necrotic cell death. However, while during PAM treatment cell number and

hormone secretion decreased in parallel, we observed specific impairment of steroidogenesis in the presence of CLO with a dose-dependent increase in the 17-OH-progesterone/cortisol ratio (100 μ M CLO $134 \pm 30\%$; 500 μ M CLO $284 \pm 10\%$; 1000 μ M CLO $545 \pm 130\%$ vs $100 \pm 20\%$ in control cells; $P < 0.01$). Further analysis in ACTH-stimulated bovine adrenal cells using stable isotope dilution/gas chromatography-mass spectrometry demonstrated CLO-induced inhibition of adrenal 21-hydroxylase (P450c21) activity leading to a dose-dependent increase in the 17-OH-progesterone/11-deoxycortisol ratio.

In conclusion, we demonstrate a dose-dependent antiproliferative effect of CLO and PAM in adrenocortical cells. In addition, for the first time, we describe a suppressive effect of CLO on steroidogenesis via inhibition of adrenal 21-hydroxylase (P450c21) activity.

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Introduction

Bisphosphonates (BPs) are analogs of pyrophosphate with a potent inhibitory effect on bone resorption (Sahni *et al.* 1993, Rodan & Fleisch 1996, Fleisch 1998, Russell & Rogers 1999). While a direct effect of BPs on osteoclasts has been repeatedly demonstrated, recent studies have reported specific effects also in other cell types like macrophages (Frith *et al.* 1997, Luckman *et al.* 1998a) and some tumor cell lines (Shipman *et al.* 1997, Aparicio *et al.* 1998, Fromiguet *et al.* 2000, Senaratne *et al.* 2000, Lee *et al.* 2001, Sonnemann *et al.* 2001). BPs have an established role in the prevention and therapy of osteolytic lesions in patients with malignant diseases like multiple myeloma (Berenson *et al.* 1996, Berenson 1997, Kanis & McCloskey 2000) or breast cancer (Hortobagyi *et al.* 1996, 1998, Lipton 2000, Pavlakakis & Stockler 2002). According to a recent Cochrane review clodronate (CLO) and

pamidronate (PAM) should be the preferred agents in this situation (Djulfbegovic *et al.* 2001). Moreover, results of some clinical trials suggest that BPs (like CLO) may have a direct antitumor effect leading to a survival advantage in patients with breast cancer (Diel *et al.* 1998, Mundy & Yoneda 1998) or multiple myeloma (Kanis & McCloskey 2000).

At the cellular level one of the actions of BPs is to induce apoptosis. Interestingly, two different molecular pathways contribute to this apoptotic activity of BPs. CLO and other non-amino BPs induce both necrotic and apoptotic cell death after metabolism of the drug to a nonhydrolyzable, but cytotoxic ATP analog (Frith *et al.* 1997, Fleisch 1998, Benford *et al.* 1999, Rogers *et al.* 2000). In contrast, the nitrogen-containing BPs, such as PAM and alendronate inhibit the intracellular mevalonate pathway enzyme, farnesyl diphosphate synthase, and have been reported to lead to apoptosis (Luckman *et al.* 1998a,b,

Reszka *et al.* 1999, van Beek *et al.* 1999a, Fisher *et al.* 2000, van Beek *et al.* 2002). In addition, other mechanisms such as effects on the cytoskeleton and membrane structures by inhibition of protein geranylgeranylation have recently been described (Coxon *et al.* 2000, 2001, Halasy-Nagy *et al.* 2001).

Adrenocortical carcinoma is a rare and highly malignant tumor with an incidence of 1:1.7 million/year (Nader *et al.* 1983). At the time of diagnosis 70% of patients suffer from advanced disease with local invasion or distant metastases (Soreide *et al.* 1992). Next to lung and liver, the skeleton is the most frequent localization of disseminated disease. The prognosis in advanced adrenocortical carcinoma is poor and new treatment options are urgently needed. At least 50% of all patients with adrenocortical carcinoma suffer from cortisol hypersecretion leading to Cushing's syndrome (Schteingart & Homan 2001).

Here we report antiproliferative activity of BPs in the human adrenocortical carcinoma cell line NCI-H295. In addition, CLO, but not PAM, specifically impairs adrenal steroidogenesis by inhibition of the 21-hydroxylase enzyme (P450c21) in NCI-H295 cells and in bovine adrenocortical cells.

Materials and Methods

Materials

CLO was obtained from Roche (Grenzach-Wyhlen, Germany), and PAM and synthetic adrenocorticotropin (ACTH₁₋₂₄) were purchased from Novartis Pharma (Nürnberg, Germany). RPMI 1640, MEM medium, fetal calf serum (FCS), horse serum (HS), bovine serum albumin (BSA) and trypan blue stain were supplied by Life Technologies (Eggenstein, Germany), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT), antibiotics, DMEM/HAM'S F12, collagenase II, desoxyribonuclease I, insulin, transferrin, selenium, glutamine, Hepes and Percoll were purchased from Sigma (Deisenhofen, Germany). All reagents used for flow cytometry were derived from Becton Dickinson (Heidelberg, Germany).

Cell culture

The adrenocortical cancer cell line NCI-H295 (Gazdar *et al.* 1990) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were available in an adherent and a suspension variant. The adherent cells were maintained in DMEM/HAM'S F12 medium supplemented with transferrin, insulin, selenium and 5% FCS and used for the MTT assays. The suspension cells were grown in RPMI 1640 medium supplemented with transferrin, insulin, selenium and 5% FCS as described previously (Fassnacht *et al.* 1998, 2000) and were used for flow cytometry analysis. Both variants of

cells were grown at 37 °C under an atmosphere of 5% CO₂/95% air. For experiments, the FCS concentration was reduced to 2%.

The primary culture of bovine adrenocortical cells was performed as described in detail by Weber and Michl (1996). Briefly, adrenal glands from 2- to 3-year-old steers were obtained from the local slaughterhouse. The tissue was dissected and cells of the zona fasciculata and zona reticularis were obtained by digestion with collagenase II and desoxyribonuclease I. To purify dissociated cells, and to remove contaminating red blood cells and cell debris, a Percoll centrifugation was performed. The final cell pellet was resuspended in DMEM/HAM'S F12 medium supplemented with 10% FCS, 5% HS and antibiotics. Cells were plated in 75 cm² flasks in a concentration of 10⁶ cells/ml. Medium was changed after 24 h to remove cell debris and unattached cells. During the following days, medium was replenished every 24–48 h. On day 3 (72 h after seeding) the experiments commenced and cells were incubated with different doses of BPs.

The colon carcinoma cell line, HT-29 (von Kleist *et al.* 1975) and the prostate carcinoma cell line, LNCaP (Horoszewicz *et al.* 1983) were obtained from ATCC. HT-29 cells were maintained in MEM medium supplemented with glutamine, Hepes and 10% FCS. LNCaP cells were grown in RPMI 1640 medium and 10% FCS. The breast cancer cell line, T47D (Westley & Rochefort 1980) was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI 1640 medium supplemented with glutamine, insulin and 10% FCS. The ovarian cancer cell line, OVCAR-3 (Hamilton *et al.* 1983) was purchased from the Cell Line Service (CLS, Heidelberg, Germany) and the cells were grown in MEM medium with glutamine and 10% FCS.

Dye exclusion assay

Cells were seeded at an initial concentration of 10⁶ cells/ml into six-well cell culture plates. Drugs were added at specified concentrations. At various time points, viability was determined by trypan blue staining (Duerst & Frantz 1985). The total number of viable cells was calculated and the percentage of viable cell recovery in the bisphosphonate-treated groups was determined by comparison with untreated control cells.

MTT assay

Cells were seeded into 96-well microtiter plates at a density of 50 000 cells/well. Twenty-four hours after inoculation, CLO (final concentration of 100–2000 µM) or PAM (1–100 µM) was added to the wells (*n*=8 replicates). The cells were then incubated for a further 72 h followed by addition of MTT reagent (5 mg/ml stock solution) in a final concentration of 0.5 mg/ml. After incubation at 37 °C for 3 h, the product was dissolved by

addition of 100 µl cell lysis buffer (isopropanol with HCl), and absorbance of the wells was measured at 570 nm using an ELISA plate reader (400 SF, SLT Lab Instruments, Crailsheim, Germany).

Flow cytometry analysis

NCI-H295 cells were cultured as described above with different concentrations of CLO (500–1000 µM) and PAM (50–100 µM) for 4–48 h. After incubation, cells were washed twice with sterile PBS containing 0.1% (w/v) BSA at room temperature. Then 1×10^6 cells were stained using 0.25 µg 7-amino-actinomycin D (7-AAD) as a nucleic acid dye and 5 µl of a phycoerythrin (PE)-conjugated antibody to annexin V at a total volume of 100 µl. After an incubation time of 10 min in the dark, 400 µl PBS containing 0.1% BSA (w/v) were added and cells were analyzed by flow cytometry. Using Cell Quest software and a FacsCalibur flow cytometer (both Becton Dickinson) 1×10^5 cells were gated in a dot plot forward scatter (FSC) versus side scatter (SSC). In a dot plot fluorescence 3 (7-AAD) versus fluorescence 2 (annexin V-PE) quadrant statistics were taken to detect double positive cells (7-AAD and annexin V, late apoptotic – effectively dead – cells) as well as cells negative for 7-AAD and positive for annexin V (early apoptotic cells). All data are given as percentage (geometric mean $\pm$ s.d.) of gated cells.

Steroid determination

Adrenocortical cells were incubated for 48 h with CLO (100–2000 µM) or PAM (1–100 µM). NCI-H295 cells were also incubated for shorter time periods (2.5 and 24 h). Each experiment was performed in triplicate. ACTH stimulation was performed only in bovine adrenocortical cells, because the human NCI-H295 cells express ACTH receptor at a very low level and these cells are, therefore, less suitable for investigating ACTH-induced effects on steroidogenesis. ACTH incubation (4 h) was performed after 48 h of pre-treatment with 0, 100, 500 or 1000 µM CLO. The media were changed and the fresh media without BPs were supplemented with 10 nM ACTH and the cells were incubated for 4 h. At the end of the incubation period steroid hormones (cortisol and 17-OH-progesterone) were determined in the cell supernatant by commercially available radioimmunoassays (DPC Biermann, Bad Nauheim, Germany). Additionally, in bovine adrenal cells after ACTH stimulation, cortisol, 11-deoxycortisol, 17-hydroxy-progesterone, 17-hydroxy-pregnenolone, androstendione, and testosterone were determined by stable isotope dilution/gas chromatography-mass spectrometry (GC-MS) as described elsewhere (Wudy *et al.* 2001). In brief, cell supernatant was equilibrated with internal standard (deuterium-labeled analogs of analytes). After solvent extraction and purification, heptafluorobutyrate derivatives were prepared. Gas

Figure 1 Dose-dependent effect of bisphosphonates on cell viability in the human NCI-H295 cell line as measured by the MTT assay. Cells were treated with 100–2000 µM clodronate (A) or 1–100 µM pamidronate (B) for 96 h. Results are given as a percentage of untreated control cells (100%) and are shown as means $\pm$ s.d. * $P < 0.01$ compared with control cells.

Figure 2 Dose-dependent effect of 96-h incubation with clodronate (100, 500 or 1000 µM) and pamidronate (10, 50 or 100 µM) on cell viability in bovine adrenocortical cells as measured by the MTT assay. Results are given as a percentage of untreated control cells (100%) and are shown as means $\pm$ s.d. * $P < 0.01$ compared with control cells.

chromatography was carried out on an Agilent 6890 series GC housing an OV-1 fused silica capillary (25 m $\times$ 0.2 mm, film 0.1 µm, Macherey-Nagel, Düren, Germany) coupled with an Agilent 5973 mass selective detector operated in the selected ion-monitoring mode. For computerized quantification, peak areas of specific ions of analytes and their corresponding internal standards were

Table 1 Effects of clodronate and pamidronate on cell viability in human adrenocortical NCI-H295 cells measured by annexin and 7-AAD staining and flow cytometry analysis. Cells were incubated for 4, 6, 24 or 48 hours with 500 or 1000 µM clodronate or 50 or 100 µM pamidronate. Cells stained only for Annexin V indicate early apoptosis, 7-AAD-positive (pos.) cells indicate necrosis. Both annexin V- and 7-AAD-positive cells indicate late apoptosis, effectively dead cells. Results (means ± s.d.) are given as percentage of all gated cells (100%)

	Viable cells	Annexin V pos. +7-AAD neg.	Annexin V pos. +7-AAD pos.	Annexin V neg. +7-AAD pos.
4 hours				
Untreated cells	85.6 ± 0.4	5.2 ± 3.7	4.0 ± 1.1	5.0 ± 4.0
1000 µM clodronate	79.2 ± 0.9*	7.6 ± 1.3	9.6 ± 0.8*	3.5 ± 0.7
100 µM pamidronate	87.7 ± 1.6	7.0 ± 2.2	3.9 ± 1.7	1.4 ± 0.7
6 hours				
Untreated cells	83.6 ± 0.3	3.5 ± 0.3	4.8 ± 1.0	7.9 ± 1.0
1000 µM clodronate	70.1 ± 1.5*	7.9 ± 0.3*	14.0 ± 0.8*	8.0 ± 1.2
100 µM pamidronate	84.6 ± 2.2	3.0 ± 1.1	6.7 ± 1.2	5.6 ± 0.6
24 hours				
Untreated cells	72.0 ± 3.2	8.2 ± 1.2	12.8 ± 2.2	6.9 ± 2.2
1000 µM clodronate	60.9 ± 5.7*	10.3 ± 1.3	20.1 ± 4.3	8.5 ± 1.5
100 µM pamidronate	55.9 ± 5.8*	9.6 ± 0.8	22.4 ± 5.5*	12.0 ± 4.4
48 hours				
Untreated cells	77.1 ± 1.9	7.7 ± 2.1	9.3 ± 0.6	5.9 ± 0.7
500 µM clodronate	62.1 ± 4.6*	12.4 ± 3.7	17.2 ± 2.2*	8.6 ± 1.9
1000 µM clodronate	38.4 ± 3.2*	14.0 ± 0.9*	32.5 ± 3.7*	15.1 ± 1.4*
50 µM pamidronate	67.3 ± 4.4*	11.3 ± 3.3	14.0 ± 2.6*	7.4 ± 1.8
100 µM pamidronate	32.1 ± 1.9*	12.9 ± 4.4*	38.7 ± 2.6*	16.2 ± 1.5*

neg., negative.
*P<0.05 compared with untreated cells.

recorded. Dehydroepiandrosterone, androstandiol and dihydrotestosterone levels were below the limit of detection of the method.

Statistical analysis

All results are expressed as means ± s.d. Significance of differences were evaluated by ANOVA (Statview 4.51, SAS Institue Inc., Cary, NC, USA). A value of P<0.05 was considered statistically significant.

Results

Cell proliferation, apoptosis and necrosis

Treatment of the adrenocortical cancer cell line NCI-H295 led to a dose-dependent decrease of cell viability as measured by the MTT assay (Fig. 1A). This effect was paralleled by a reduced cell number as determined by trypan blue staining after an incubation time of 48 h (100 µM CLO: 99 ± 12%, 500 µM CLO: 82 ± 11%, 1000 µM CLO: 66 ± 9% compared with untreated control cells: 100 ± 10%; P<0.01). Incubation of the cells with the amino BP PAM caused a comparable decrease in cell viability but at much lower concentrations (Fig. 1B). Similar dose-dependent antiproliferative effects were detected in bovine adrenocortical cells (Fig. 2). To exclude a non-specific toxic effect, we evaluated the cell viability in the MTT assay in four different non-adrenal cancer cell lines after incubation with

1000 µM CLO. Significant suppression of cell viability was seen in the breast cancer cell line T47D (1000 µM CLO 38 ± 5% compared with untreated control cells 100 ± 7%; P<0.01) and in the ovarian carcinoma cell line OVCAR-3 (1000 µM CLO 83 ± 4% compared with untreated control cells 100 ± 9%; P<0.01). No reduction in cell viability was seen in the colon carcinoma cell line HT-29 (1000 µM CLO 115 ± 6% compared with untreated control cells 100 ± 7%) and in the prostate cancer cell line LNCaP (1000 µM CLO 102 ± 11% compared with untreated control cells 100 ± 15%).

As BPs are known to induce both apoptosis and necrosis in different cells, we analyzed translocation of phosphatidylserine on the outer cell membrane as a sign of apoptosis in NCI-H295 cells by flow cytometry. Forty-eight hours of incubation with CLO or PAM in NCI-H295 cells led to a dose-dependent increase of annexin V- and 7-AAD-staining cells analyzed by flow cytometry (Table 1) indicating apoptotic and necrotic cell death. Similar results were seen after 4, 6 and 24 h (Table 1).

Hormone secretion

In both NCI-H295 cells and bovine adrenocortical cells, both CLO and PAM led to a dose-dependent decrease in cortisol secretion. However, further analysis demonstrated different effects of CLO and PAM on steroidogenesis. PAM reduced cortisol and 17-OH-progesterone secretion in parallel with the decrease in cell number with no

Figure 3 Effects of clodronate (A) and pamidronate (B) on cell viability and hormone secretion in human NCI-H295 cells. Cells were incubated for 96 h with increasing doses of the bisphosphonates as indicated. Cell viability was measured by MTT assay; cortisol and 17-OH-progesterone (17-OH-P) secretion were determined in the cell supernatant by radioimmunoassay. Results are given as percentage of untreated control cells (100%) and are shown as means $\pm$ S.D.

change in the 17-OH-progesterone/cortisol ratio (Fig. 3B). In contrast, cortisol secretion in CLO-treated cells was suppressed, while 17-OH-progesterone secretion remained unaffected or was even increased (Fig. 3A). A similar pattern was seen after 2.5 and 24 h of incubation. In PAM-treated cells the 17-OH-progesterone/cortisol ratio remained unaffected, while it was significantly increased in cells treated with 1000 μ M CLO in comparison with untreated control cells (2.5 h: 125 ± 17 vs $100 \pm 8\%$; 24 h: 139 ± 17 vs $100 \pm 5\%$; both $P < 0.05$) although this increase was clearly smaller than after long-term incubation (48 h). To further elucidate the CLO-induced inhibition of steroidogenesis we investigated, in more detail, steroid secretion after ACTH stimulation

(4 h) via GC-MS in bovine adrenocortical cells pre-treated with CLO (48 h). In comparison with control cells not pre-treated with CLO, the ACTH-stimulated 17-OH-progesterone secretion in CLO pre-treated cells was dramatically increased while cortisol secretion increased only slightly. Deoxycortisol secretion increased only slightly, in parallel with cortisol (Table 2 and Fig. 4) indicating inhibition of P450c21 enzyme activity by CLO.

Discussion

Our study shows a clear antiproliferative activity of both the non-amino BP CLO and the amino BP PAM in the human adrenocortical cancer cell line NCI-H295 and in primary culture of bovine adrenocortical cells. An intriguing finding was the unexpected observation of a differential effect of PAM and CLO on steroidogenesis: while the amino BP PAM reduced both cortisol and 17-OH-progesterone secretion in parallel with its antiproliferative activity, there was evidence for specific inhibition of adrenal 21-hydroxylase activity by CLO. This was demonstrated in both NCI-H295 cells and in primary cultures of bovine adrenocortical cells by an increased 17-OH-progesterone/cortisol ratio.

Direct antiproliferative activity of BPs has been reported recently in a variety of tumor cell lines (Shipman *et al.* 1997, Aparicio *et al.* 1998, Busch *et al.* 1998, Fromigue *et al.* 2000, Senaratne *et al.* 2000, Lee *et al.* 2001, Sonnemann *et al.* 2001). However, as has been observed in this study for the colon cancer cell line HT29 and the prostate cancer cell line LNCaP, not all tumor cells are equally sensitive to antiproliferative effects of BPs, suggesting specific mechanisms of action. Possibly the ability to internalize bisphosphonates is different in these cell lines. Moreover, there is evidence that amino BPs and non-amino BPs exert differential effects in both osteoclasts and tumor cells. Frith *et al.* (1997) demonstrated that the non-amino BP CLO can be metabolically incorporated into a nonhydrolyzable analog of ATP in the cytoplasm of J774 macrophages *in vitro*. The intracellular accumulation of this metabolite may be responsible for the observed growth inhibitory effect. This cytotoxic mechanism has also been demonstrated for other non-amino BPs (Benford *et al.* 1999) and leads to a combination of apoptosis and necrosis (Fromigue *et al.* 2000). Flow cytometry analysis in our study also indicated such a combination of apoptosis and necrosis in adrenocortical cancer cells after incubation with CLO. Although flow cytometry analysis is not the gold standard to differentiate between apoptosis and necrosis, the increased number of annexin-positive cells after incubation with CLO and PAM clearly suggests an involvement of apoptosis. In contrast, amino BPs like PAM induce apoptosis by inhibition of the mevalonate pathway with consecutive impairment of protein prenylation (Luckman *et al.* 1998a,b, Benford *et al.* 1999, Reszka *et al.* 1999, van Beek *et al.* 1999a,b, Fisher *et al.* 2000).

Table 2 Steroid release in bovine adrenocortical cells pre-treated with clodronate after ACTH stimulation. Cells were pre-treated with or without clodronate (100–1000 µM) for 48 h, medium was changed and cells were incubated with 10 nM ACTH for 4 h. Different steroid hormones were determined in the cell supernatant by stable isotope dilution/gas chromatography-mass spectrometry. Hormone levels (means ± S.D.) are given in ng/10⁶ cells/4 h

Pre-treatment	Cortisol	Deoxycortisol	17-OH-progesterone	17-OH-pregnenolone	Androstendione	Testosterone
Control	965.6 ± 30.9	302.5 ± 14.5	47.7 ± 5.2	10.9 ± 0.6	31.5 ± 3.8	0.81 ± 0.11
100 µM CLO	1223.2 ± 50.1*	522.4 ± 6.8*	147.9 ± 19.9*	7.57 ± 0.6*	43.5 ± 7.5	1.23 ± 0.34
500 µM CLO	1754.6 ± 87.9**	665.8 ± 6.3**	477.4 ± 67.1**	13.0 ± 1.4	54.9 ± 12.3*	1.42 ± 0.29*
1000 µM CLO	1900.4 ± 88.6**	752.9 ± 35.6**	568.8 ± 44.6**	16.3 ± 1.6**	47.4 ± 9.4	1.45 ± 0.1*

*P<0.05; **P<0.01 compared with controls.

However, the increased number of 7-AAD-stained cells without any annexin staining suggested that necrosis also contributes to the antiproliferative activity of CLO and PAM in NCI-H295 cells.

In adrenocortical cells the differential effect of the amino BP PAM and the non-amino BP CLO was most strikingly evident from the differential effects on steroidogenesis. NCI-H295 cells are the most suitable model for studying steroidogenesis, since this cell line is the only available human cell line which expresses all major adrenal steroidogenic enzymes and has the ability to produce all the adrenocortical steroids (Gazdar *et al.* 1990, Staels *et al.* 1993, Rainey *et al.* 1994). Due to the diminished response

of the NCI-H295 cells to ACTH (Rainey *et al.* 1993, Mason *et al.* 1995), we analyzed the changes in the hormone secretion profile after ACTH stimulation in primary cultures of bovine adrenocortical cells. It has been shown that these cells produce large amounts of steroid hormones after incubation with ACTH (Weber & Michl 1996, Le Roy *et al.* 2000). In both cell types only CLO exhibited a suppressive activity on cortisol secretion beyond mere reduction in cell number. Further analysis using stable isotope dilution/GC-MS clearly established inhibition of adrenal 21-hydroxylase (P450c21) activity as the cause of the increased 17-OH-progesterone/cortisol ratio (Fig. 4). This effect is already evident at low concentrations (100 µM), which do not affect cell viability. This inhibition of the adrenal 21-hydroxylase enzyme was reversible after removal of CLO. After addition of fresh medium (without CLO) CLO pre-treated cells showed a higher cortisol response to ACTH than control cells. This is most likely due to partial recovery from enzyme inhibition and the CLO-induced increase in cortisol precursors.

In contrast, steroid secretion in PAM-treated cells strictly paralleled cell number without evidence of specific inhibition of steroidogenic enzymes.

It remains unclear by which mechanism CLO induces the observed inhibition of the adrenal 21-hydroxylase enzyme. The fact that short-time incubation with CLO was less inhibitory may indicate that inhibition of steroidogenesis is mediated indirectly or by metabolites of CLO. It is known that adrenal P450 steroidogenic enzymes are under the control of the ACTH-induced cAMP–protein-kinase A pathway (Staels *et al.* 1993). Thus, by generating a nonhydrolyzable analog of ATP CLO may impair this signaling cascade. However, it would be expected that impairment of the cAMP system would affect all steroidogenic enzymes. Thus, either the 21-hydroxylase enzyme is more sensitive to changes in the cAMP-system or CLO acts via another mechanism, possibly also by directly interfering with P450c21 activity.

Our findings may have clinical implications. CLO may be a useful adjuvant treatment in patients with adrenocortical carcinoma and bone metastases. Several studies have found that CLO serum concentrations reach 30 µM after a

Figure 4 Effect of pre-treatment with clodronate on ACTH-induced hormone secretion in bovine adrenocortical cells. Cells were pre-treated with or without clodronate (100–1000 µM) for 48 h, medium was changed and cells were incubated with 10 nM ACTH for 4 h. Cortisol, 11-deoxycortisol, and 17-OH-progesterone secretion were determined in the cell supernatant by stable isotope dilution/gas chromatography-mass spectrometry. Results (means ± S.D.) are given as a percentage of hormone secretion of cells not pre-treated with clodronate (100%).

single intravenous dose of 300 mg (Yakatan *et al.* 1982, Hanhijarvi *et al.* 1989, Hurst & Noble 1999). Ylitalo *et al.* (1999) demonstrated that CLO concentrations in serum after orally administered CLO increase dose-dependently. During the maintenance therapy in cancer patients the renal drug clearance seems to be lower than in healthy volunteers (Ylitalo *et al.* 1999). Moreover, in patients with osteolytic metastases, high doses of CLO (up to 2000 mg orally daily or 1500 mg i.v. every 14–21 days) are given, so that peak serum levels of 200–300 µM are likely. In addition, it has been suggested that the effective local concentration of BPs at the site of active bone resorption is much higher than corresponding serum levels due to their high affinity for bone mineral (Sato *et al.* 1991).

At present, it is not clear whether inhibition of steroidogenesis by CLO is also relevant *in vivo* in patients receiving CLO. Two of us (M F and A F) volunteered to receive an infusion of a single dose of 1500 mg CLO combined with short ACTH tests (250 µg ACTH_{1–24}) prior to and after CLO infusion. After the CLO challenge an increase in the 17-OH-progesterone/cortisol ratio after ACTH stimulation was observed in both cases (to 163% and 133% respectively of values prior to CLO infusion). However, as the cortisol response to ACTH was not reduced, this may indicate that, *in vivo*, significant impairment of adrenocortical function by CLO therapy is unlikely. On the other hand, long-term treatment may lead to chronic exposure and more pronounced impairment. Moreover, some patients may be more susceptible to the inhibitory activity of CLO (e.g. heterozygotes for congenital adrenal hyperplasia), and interaction with other treatment options in tumor patients (e.g. ketoconazole) may lead to impairment in the adrenal stress response. Thus further studies are required to elucidate the effects of chronic CLO treatment on adrenal function in tumor patients.

In summary, we could demonstrate a dose-dependent antiproliferative effect of CLO and PAM in adrenocortical cells. In addition, for the first time, we describe a specific suppressive effect of CLO on steroidogenesis via inhibition of the adrenal 21-hydroxylase enzyme (P450c21) activity.

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