The antiproliferative effects of ouabain and everolimus on adrenocortical tumor cells

Raffaele Pezzani¹), Beatrice Rubin¹), Marco Redaelli²), Claudia Radu³), Susi Barollo¹), Maria Verena Cicala¹), Monica Salvà¹), Caterina Mian¹), Carla Mucignat-Caretta²), Paolo Simioni³), Maurizio Iacobone⁴) and Franco Mantero¹)

¹)Endocrinology Unit, Department of Medicine, University of Padova, Padova 35128, Italy
²)Department of Molecular Medicine, University of Padova, Padova 35131, Italy
³)Department of Cardiologic, Thoracic, and Vascular Sciences, 2nd Chair of Internal Medicine, University of Padova Medical School, Padova 35128, Italy
⁴)Endocrine Surgery Unit, Department of Surgical and Gastroenterological Sciences, University of Padova, Padova 35128, Italy

Abstract. Ouabain is a cardiotonic steroid obtained from Strophanthus. Recently its role as antiproliferative agent has been investigated in tumor cells. Everolimus is a derivative of rapamycin and acts as a signal transduction inhibitor. Adrenocortical carcinoma is a rare cancer, with poor prognosis. This research focuses on antineoplastic properties of ouabain and its association with everolimus. We analyzed the effects of drugs on cells by MTT assay, by [³H] thymidine assay, by Wright’s staining, by homogeneous caspases assay, by flow cytometry analysis and by Western blot analysis on H295R and SW13 cells and on primary adrenocortical tumor cells. Ouabain induced cell viability reduction in SW13, H295R and 5 primary adrenocortical tumor cells. Combination of ouabain with everolimus produced a stronger cytotoxic effect on cell proliferation and viability. Marked morphological changes were observed in both SW13 and H295R cell lines after ouabain treatment, with an increase in necrosis. Cell cycle distribution was altered by ouabain in SW13. Analysis of apoptosis demonstrated an increase in caspase activity, clearly evident for SW13 at 72h. FACS analysis by Annexin V-FITC kit and propidium iodide confirmed an increased level of necrosis at higher concentrations. Western blot analysis showed that PI3k/Akt signaling pathway was modified after ouabain treatments in SW13. Ouabain exerts antiproliferative effects on SW13 and H295R cell lines and on primary adrenocortical tumor cells. These data suggest that ouabain or ouabain derivatives may be potential anticancer agents.

Key words: Ouabain, Antiproliferative, Adrenocortical tumor, Primary cell cultures, Everolimus

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Everolimus (RAD001) is a derivative of rapamycin acting on the mammalian target of rapamycin (mTOR), a key component of PI3K/Akt pathway, frequently activated in human cancers. It is a well-known compound currently used for the treatment of renal cell cancer and other tumors [8]. Recently we described its antiproliferative effect in adrenocortical tumor models in association with sorafenib [9].

Adrenocortical tumors can be benign or malignant. Benign tumors are frequent in the general population, being commonly discovered incidentally (incidentaloma) and may be functional or nonfunctional. Malignant adrenocortical carcinoma (ACC) has a poor prognosis with an estimated survival rate of 35% at 5 years, and to date pharmacologic therapy is principally based on the adrenotoxic drug, mitotane (o,p'-DDD), despite its low response rate and significant toxicity [10]. As this regimen is insufficient, it is necessary to develop new treatment modalities.

To determine whether ouabain alone, or in combination with everolimus, exerts antineoplastic activity, we explored its effects on cell viability, cell proliferation, cell morphology, cell cycle and apoptosis in SW13 and H295R cells (the 2 most frequently used adrenocortical cell models), and in primary adrenocortical tumor cells.

Materials and Methods

Materials
Ouabain, everolimus, fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trichloracetic acid (TCA) and propidium iodide were purchased from Sigma Aldrich (Sigma Aldrich Corporation, St. Louis, MO, USA). DMEM-F12, 0.05% trypsin-EDTA, insulin, transferrin, selenium and antibiotics were from Invitrogen (Invitrogen S.R.L. Milano, Italy). Primary antibodies were: anti-Akt (cod. 9272), anti-phospho-Akt (Ser473) (cod. 9271), anti-P70S6K (cod. 2708), anti-phospho-P70S6K (Thr389) (cod. 9234) all from Cell Signaling Technology and mouse monoclonal anti-β-Actin antibody (cod. A5441) from Sigma-Aldrich. Secondary antibodies were: horseradish peroxidase-labeled goat anti-mouse or anti-rabbit (Jackson ImmunoResearch laboratories Inc., West Grove, PA, USA).

Patients
Adrenocortical tumor tissues were obtained from 1 patient with ACC and 4 patients with aldosterone-producing adenomas (APA). The etiology of each adrenocortical mass, clinical diagnose and staging were established as described [9]. All clinical data are summarized in Table 1. All patients gave written informed consent to the collection and use of adrenal tissue for research purposes, and the study was approved by the local ethics committee (conforming to the Declaration of Helsinki, revised in Tokyo, 2004).

Cell cultures
H295R and SW13 adrenocortical tumor cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). H295R cells were established from a female patient diagnosed with an adrenocortical carcinoma. This strain secretes mineralcorticoids, glucocorticoids and adrenal androgens. SW13 cells were derived from a small cell carcinoma in the adrenal cortex of a 55-year-old female, probably a metastasis to the adrenal cortex. This strain produces no steroid [11]. Five primary cell cultures were also studied, 1 ACC and 4 APA. All experiment involving cell manipulation were conducted incubating cell lines with 0.1% FBS as previously described [12].

MTT assay
Cells were plated in 96-well plates at a density of 5x10^3 cells/well in supplemented medium with or with-

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M = Male; F = Female; A = aldosterone; C = cortisol; Exp1=MTT test, Exp2=Wright’s staining, Exp3=flow cytometry analysis, NA=Not applicable.
Ouabain and everolimus in adrenal

**Homogeneous caspases assay**

H295R and SW13 cells were plated into 96-well plates at a density of 2x10^4 cells/well and were treated with ouabain (1, 10, 100, 1000 nM) for 24h and 72h. Total caspase activity was evaluated with a Homogeneous Caspases Assay Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer’s instructions. In brief, substrate solution was added to cell lysate for 2h at 37°C and relative fluorescence was measured with excitation and emission filters (λex=499nm and λem=520nm). Data were corrected by subtracting the blank absorbance and concentrations estimated against a calibration curve obtained with R110 standard solutions. Each experiment was performed in triplicate and repeated twice.

**Apoptosis flow cytometry analysis**

Apoptosis was determined by Annexin V-FITC kit (BD Biosciences, San Diego, CA, USA). In brief, H295R and SW13 cells were plated into 25 cm^2 flasks at a density of 1x10^6 cells/well for two days, then maintained overnight at 0.1% FBS. The day after the cells were treated with ouabain alone (1, 10, 100, 1000 nM) for 24 and 72h or in combination with everolimus (5 µM), trypsinized and harvested by centrifugation. Cells were then stained with Annexin V-FITC and propidium iodide according to the manufacturer’s instruction. Flow cytometry analysis was also performed on 2 primary adrenocortical tumor cells: APA-1 and ACC. The concentrations of ouabain used were 1, 10, 100, 1000 nM for 24h or 72h (and 120h for APA-1 primary tumor cell culture).

**Western blot analysis**

Cells were treated with different concentrations of ouabain 1, 10, 100 nM at 24h for SW13 cells and 1, 10, 100, 1000 nM at 24h for H295R cells as previously described [9]. In addition everolimus (5 µM) was used in combination with ouabain. Briefly proteins were extracted with lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich) for 1h at 4°C, loaded onto SDS/PAGE, electroblotted onto nitrocellulose mem-
Ouabain alters cell morphology in SW13, H295R and primary adrenocortical tumor cells

To evaluate cell morphology, Wright’s staining method was used. As shown in Fig. 3, apoptosis, determined by blebbing, cell shrinkage, nuclear fragmentation, condensation of chromatin and necrosis, was determined by cell disruption and membrane breakdown, both of which were clearly detected. Marked morphological changes were observed both for SW13 and H295R cells (Fig. 3A-D). In H295R, SW13 and in primary adrenocortical tumor cells (APA-1), necrotic processes were similarly predominant over apoptotic processes (data not shown).

Ouabain induces cell cycle deregulation in SW13 cells

To study the cell cycle distribution, PI assays were performed. In H295R cells, ouabain treatment alone or in combination produced no effect at 24h, but at 72h a slight G0/G1 phase increase after ouabain treatment alone (1000nM) or combination regimen (ouabain 1000nM + everolimus 5µM) was detected (data not shown). In SW13 cells, ouabain treatment promoted a G0/G1 phase increase both at 24h and 72h, with a concomitant S and G2/M phases reduction, particularly evident at 100 and 1000nM at 72h (Fig. 4A). Combination treatment produced no change in G0/G1 phase, a slight increment in S phase and a slight decrease in G2/M phase, especially evident when ouabain 1000nM + everolimus 5µM was used (Fig. 4B).

Ouabain affects caspase activity in SW13 and H295R cells

To assess the first steps of apoptotic process, the activation of a particular group of proteases, the caspases, was analyzed by a specific assay in both cell lines. The activity of caspases evaluated at 24h are not dissimilar to basal for both cell lines, despite a statistical difference (Fig. 5A-B). The effect of ouabain (10, 100 and 1000nM) on caspase activity proved to be more marked at 72h for both cell lines; in SW13 cells, caspase activity clearly increased at 100 and 1000nM ouabain at 72h (Fig. 5B).

Ouabain does not induce apoptosis in SW13, H295R and in primary adrenocortical tumor cells

To discriminate between apoptosis and necro-
Fig. 1 Cell viability of adrenocortical tumor cells estimated by MTT assay. A and B, cell viability in SW13 and H295R at 24h and 72h of treatment. C and D, cell viability in SW13 and H295R at 24h and 72h treated with ouabain (O) and everolimus (E). Primary adrenocortical tumor cells were treated with ouabain: E, cell viability in ACC primary tumor cells at 24h and 72h; F, cell viability in APA-1 primary adrenocortical tumor cells at 24h, 72h and 120h. Treatment vs control:* p < 0.05. Each analysis was performed in 4 replicates and repeated in 3 independent experiments.
Fig. 2 Proliferation rate of SW13 and H295R cells at 24h and 72h of ouabain treatment assessed by [\(^3\)H] thymidine incorporation assay. The results are expressed as a percentage of control (100%). Treatment vs control: * \(p < 0.05\); ** \(p < 0.005\); *** \(p < 0.001\). Each experiment was performed in quadruplicated and repeated 3 times.

Fig. 3 Cell morphology of SW13 and H295R at 24h evaluated by Wright’s staining method. A-D, panel of 4 representative observations: A and B, Wright’s staining of SW13 (control and 1000nM), C and D, Wright’s staining of H295R (control and 1000nM). Black arrows: necrotic cells, white arrows: apoptotic cells. Each experiment was performed 3 times.
Fig. 4 Representative distribution of cell cycle analysis of SW13 cells at 72h of ouabain alone or in combination with everolimus 5µM performed by flow cytometric analysis with propidium iodide. A, treatment with ouabain (1, 10, 100, 1000nM); B, combination treatment (E=everolimus, O=ouabain). Representative distribution of cell cycle analysis, performed in triplicates.

Fig. 5 Caspase activity (as free R110) of SW13 and H295R cells at 24h and 72h of treatment was evaluated by homogeneous caspase assay. A, H295R cells. B, SW13 cells. Treatment vs control:* p < 0.05; ** p < 0.005; *** p < 0.001. Each experiment was performed in triplicate and repeated 2 times.
sis, Annexin V-FITC/propidium iodide staining and flow cytometry were performed in both cell models and 2 primary adrenocortical tumor cells (1 ACC and APA-1). In SW13 cells, we observed a clear increase in necrotic processes at 1000nM after 24h (data not shown). At 72h we detected a very toxic effect of ouabain especially at 100 and 1000nM with high levels of necrosis (Fig. 6A-B), very similar to combination treatment (Fig. 6C-D). No appreciable effect was evident for apoptosis or necrosis in H295R cells at 24h and 72h (both with ouabain alone or combination treatment), and in ACC primary tumor cells, ouabain exerted a slight necrotic effect at 1000nM, with no effect evident in APA primary cells (data not shown).

**Ouabain and everolimus suppress PI3k/Akt signaling pathways in SW13 cells**

To evaluate the PI3k/Akt signaling pathway involved in apoptosis, survival and proliferation, Western blot analysis was done on both cell lines. No substantial change was observed for H295R cells, except for an absence of reactivity for phosphorylated p70S6k, both in ouabain (Fig. 7A) and combination treatments (Fig. 7C). An ouabain concentration dependent effect was particularly evident at 1000nM drug treatment. The results were different for SW13 cells, which showed a decrease in Akt, p-Akt, p70S6k and p-p70S6k reactivity after ouabain administration (Fig. 7B) or after combination regimen (Fig. 7D). Band quantification statistical analysis are in Supplemental Tables 1, 2.
Ouabain and everolimus in adrenal

To our knowledge, this is the first time that antiproliferative effects of ouabain have been investigated in an established adrenocortical cell line (H295R), in a cell line from a suspected adrenal metastasis (SW13) and in 5 primary adrenocortical tumor cells. We used H295R cell line as an effective model of adrenocortical tumor: this line requires a longer population doubling time >72-96h and produced adrenal steroids, whereas SW13 cells show a rapid duplication time of 36-48h [11]. In the literature no data are available about the therapeutic range of ouabain in serum, even though Clark and collaborators found a range of 0.6-3.2nM for digoxin, which is a CS similar to ouabain in structure and properties [13].

Based on this information and on the fact that to block Na⁺/K⁺-ATPase lethal concentrations of ouabain (>10⁶M) are needed, we sought to treat cells from 1nM to 1000nM. In combination strategy we used everolimus at 5µM, a concentration already tested in adrenocortical tumors [9].

The present study demonstrated that ouabain alone or in combination with everolimus induced a decrease in cell viability, as shown by MTT assay. The activity of ouabain was more pronounced in SW13 cells and seemed to be time and concentration dependent (Fig. 1A-E). In H295R cells the efficacy of ouabain seemed to be related to time of treatment, as only at 72h was it possible to reach the IC₅₀ (Fig. 1A). The difference between the 2 cell lines might mainly reflect the longer doubling time of H295R than SW13, even if other cell-intrinsic causes (biochemical/cytological) are also involved; in fact H295R cells carry mutations in different genes, such as CTNNB1, TP53, etc. [15].

These data are in line with previous findings, where cytotoxic effect of ouabain in a panel of cancer cell lines were seen at an IC₅₀ ranging from 50-100nM [16],
It is noteworthy that combination therapy (ouabain 10-100nM + everolimus5µM) proved to be more successful in term of cell viability reduction in H295R cells at 72h compared with treatment alone (Fig. 1C).

Primary adrenocortical tumor cells showed no change in cell viability at 24h, but at 120h for APA-1 sample, and in some measure at 72h for another APA sample, we noted a sharp decline of cell viability (70-80%) at 1000nM, probably indicating that ouabain has enough time to act on growing cells (Fig. 1F) and underlining the importance of protracted treatment.

The [3H] thymidine incorporation assay confirmed that ouabain interferes with cell proliferation, thus causing growth arrest (Fig. 2). A stochastic fall is clearly visible between 10nM and 100nM ouabain, suggesting that drug treatments are not faithfully following a sigmoidal curve. Ouabain treatment at 100nM and 1000nM may imply an interference with DNA synthesis, but not with cell viability, as at 100nM (especially in H295R cell) about 50% of cells were still viable by MTT test.

Taken together, all the above results suggest that ouabain may exert antiproliferative effects on tumor cell lines, especially on SW13 cells, even if not at lower concentrations. One previous study reported that ouabain at very high concentration (1 and 10 µM) showed antiproliferative results on 3 different prostate cancer cell lines [14]. A second study described the effects of ouabain (from 0.01 to 100µM) on viability on a small panel of cancer cell lines with essentially the same results [18]. Furthermore combination strategy seemed to be more successful in H295R cells, at least in cell viability and proliferation assay.

Examination of cells by microscopy allows accurate distinction between necrosis and apoptosis [19]. Wright’s staining results clearly indicated that necrotic processes predominated over apoptosis (Fig. 3). The DNA cell cycle distribution was altered by ouabain only in SW13 cells, while combination treatment revealed no substantial change in both cell lines. In agreement with our data on ouabain, a previous study analyzed 2 different human hepatocellular cell lines for cell cycle distribution at 24h, after treatment with ouabain at 100 and 500 nM [20].

To discriminate between the two different cellular fates, apoptosis and necrosis, and to confirm microscopy data, we performed the homogeneous caspase assay and FACS analysis by Annexin V and propidium iodide. The first method analyzes the activation of different caspases: 2, 3, 6, 7, 8, 9 and 10, one of the initial intracellular biochemical events during apoptosis [21].

It is noteworthy that the usual subdivision of necrosis versus apoptosis is a simplification of a more intricate picture. In fact cell death represents contributions by different cell processes, in which apoptosis and necrosis are overlapping phenomena; such overlapping cell death pathways are common behavior rather than an anomaly [22]. We found activation of caspases (indicative of apoptosis), especially at 72h in SW13 cells, but a major increase in necrosis was found, clearly revealed by cell morphological evaluation (Fig. 3) and FACS analysis with Annexin V-FITC and propidium iodide (Fig. 6).

These results are not in contrast, because: a) the activation of caspases is an early event in cell apoptosis, but after ouabain triggers apoptosis, cells may not be able to complete the process and subsequently undergo death via necrosis [23]; b) some caspases (1 and 8) have been observed as driving events toward necrosis and consequently ouabain may activate this process via caspase [23]; c) as previously mentioned, the two different processes may be simultaneously present in a dying cell; d) the translocation of phosphatidylserine to the outer leaflet of cell membrane (evaluated by FACS analysis) may be uncoupled from caspase activation and consequently the 2 processes may occur at different times [24]. Moreover a combination regimen seemed not differently affect cell fate, since the results are similar to those obtained with the ouabain alone. Further studies are needed to discriminate between different modalities of cell death in adrenocortical cell lines.

We analyzed the PI3k/Akt signaling pathways. Constitutive activation of this pathway is found in many malignancies and also in H295R and SW13 cells, as demonstrated in our previous work [9]. In H295R cells, ouabain induced a reduction in p-p70S6k. On the one hand this effect may be associated with decreased proliferation and survival, as underlined by [3H] thymidine assay, FACS analysis and homogeneous caspase assay. On the other, it may suggest that a specific molecular mechanism is modulated by ouabain between Akt and p70S6k, although more research is needed to confirm this hypothesis. As expected, everolimus inhibited p-p70S6k both alone or in combination with ouabain. In SW13 cells, we observed a more general effect, probably indicating that ouabain exerts its consequences on the entire pathway. Blocking PI3k/Akt signaling pathways by ouabain may be therapeutically attractive, as it was for everolimus, but this speculation needs to be adequately corroborated.
Our study has shown for the first time antiproliferative effects of ouabain on SW13 and H295R cell models and on primary adrenocortical tumor cells. The results obtained are promising, but more data are needed to fully explore the role of ouabain as a potential anticancer drug.

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Contribution Statement

We declare that all the listed authors have participated actively in the study and all meet the requirements of the authorship. R. Pezzani, B. Rubin, M. Redaelli, C. Radu, S. Barollo did the lab work. MV. Cicala and M. Salvà contributed to the literature search. R. Pezzani, M. Redaelli and F. Mantero designed the study. M. Redaelli, R. Pezzani and C. Mucignat-Caretta undertook the statistical analysis. P. Simioni, C. Mian, C. Mucignat-Caretta, M. Iacobone and F. Mantero corrected the draft and directed the work. R. Pezzani wrote the draft of the manuscript and completed the study.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

Supplemental Table 1  H295R cells

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