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Review

Ouabain as an anti-cancer agent?

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ABSTRACT

The cardiac glycoside ouabain, a well-known inhibitor of the Na/K ATPase pump, is also known to activate different cell signaling pathways that are unrelated to pump inhibition. Ouabain has, for many years, been used for congestive heart failure, but it has been suggested that its effects can be extended to other diseases. Drug repositioning of substances already in use for other indications is less time and cost consuming. The present work reviews what is known about ouabain and cancer. A wide concentration range, from pM to mM, has been studied in various experimental models. These models considered ouabain's direct action on tumor cells, effects on the inflammatory and immune responses, as well as the tumor microenvironment.

KEYWORDS: ouabain, Na/K ATPase, inflammation, immune response, tumor, cancer, cardiotonic steroid, glycosides.

1. Introduction

The process of discovering new drugs is known to be quite time-consuming and expensive. In this sense, Nishimura and Hara [1] proposed that drug repositioning, the process of identifying new indications for existing drugs, can reduce the time and cost of this process because it takes advantage of drugs already in clinical use for other indications or drugs that have cleared phase I safety trials but have failed to show efficacy for the intended diseases.

An example of this possibility are the cardiac glycosides (or digitalis or cardiotonic steroids).

1.1. Cardiotonic glycosides and ouabain

In 1775, William Whitering formally introduced the use of *Digitalis purpurea* plant extracts in the treatment of congestive heart failure with edema [2]. The term digitalis is only correctly used for glycosides obtained from *Digitalis purpurea* and *D. lanata*. However, this name is also given to cardiotonic glycosides obtained from other sources. Amongst them stands out ouabain, whose structure consists of a sugar (L-rhamnose) linked to a steroid nucleus (ouabagenin). Due to its high degree of solubility in aqueous solutions, ouabain is the most widely used glycoside in scientific research. Moreover, though ouabain is very little absorbed by oral administration, it is very efficient when administered parenterally [3].

The basis of digitalis use in congestive heart failure has both vagal and extravagal components, as digitalis substantially decreases efferent sympathetic nerve activity to the muscle in patients with heart failure [4-6]. However, the binding to the extracellular domain of the α-subunit of sodium-potassium ATPase (Na,K-ATPase), promotes inhibition of Na^+ and K^+ ion flow leading to a hydroelectrolytic imbalance. In the cardiac muscle, Na+ increase affects the $Na^+/Ca2^+$ exchanger and leads to $Ca2^+$ accumulation

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in the cytosol, generating a positive inotropic effect [7], though it seems that other proteins may directly interact with ouabain, as well as other digitalis [for a review, see 8]. In addition, it has been observed that ouabain and other digitalis promote several other effects on other organs and tissues, like the increase of vascular tone in the smooth muscle of veins, arteries and gastrointestinal tracts and also the transport of several ions along the renal tubule, promoting the transport of sodium, potassium, calcium, magnesium and inorganic phosphate, thus promoting ipsilateral diuresis [9-11].

1.2. Endogenous ouabain

The clinical effects evoked by ouabain suggested the existence of a similar compound circulating in mammals, leading to an effort to isolate and characterize the so-called endogenous ouabain (EO) in mammals. In 1991, Hamlyn and colleagues first identified this compound in human plasma, which was structurally and functionally indistinguishable from ouabain extracted from plants [12]. In the following year, Ludens and co-workers detected the presence of EO in the plasma and whole blood of rats, in addition to several other tissues [13, 14].

Despite that, there was a great controversy about the existence of EO, especially owing to the presence of L-rhamnose, a sugar considered exclusively of plant origin that would be an impediment for the physiological production of this glycoside. Nevertheless, further studies have demonstrated the synthesis of L-rhamnose in rabbit skin, bovine hypothalamus, human plasma, as well as adrenal, pituitary gland, hypothalamus and plasma from rats, thus proving that mammals are capable of synthesizing this sugar, and consequently EO [15].

Elevated levels of EO were found in the adrenal cortex, suggesting that this region could be the main source of EO production [13]. Corroborating this finding, experiments showed that adrenalectomized rats had levels of endogenous ouabain about 50% lower than normal, while demedulectomized rats maintained normal levels of EO. Moreover, primary cultures of adrenocortical cells demonstrated the secretion of this glycoside in basal conditions and after stimulation by several endogenous modulators [16], confirming that the adrenal cortex is the main source of EO.

The hypothalamus has also been described as another site of EO production. Studies evaluating the effects of destruction of sympathetic neurons in the CNS revealed a significant reduction in the hypothalamic and EO plasma levels (about 8 and 3 times, respectively), a fact that does not occur when peripheral sympathetic neurons are destroyed [17]. Moreover, hypertensive animals have hypothalamic EO levels about 10 times higher than their normotensive controls and present increased expression of genes related to ouabain biosynthesis in the hypothalamus, but not in the adrenals [18].

The levels of EO found in the plasma of healthy humans vary according to the experimental protocol applied, from the picomolar to the nanomolar range (approximately 7 nM). However, there are several physiological and pathological situations in which EO plasma levels may increase in plasma, like pregnancy and intense exercise, the latter with concentrations surpassing 100 nM [19 -21].

1.3. Ouabain and cell activation

Although Na,K-ATPase is classically described as a pump, decades ago this enzyme was also shown to communicate with the nucleus *via* activation of cell signaling pathways. Initially, this relationship was thought to rely on the inhibition of its transport activity, but a new role has been brought to light through pioneering works of Amir Askari, Zijian Xie and co-workers. They described how sub and nanomolar concentrations of ouabain were able to affect a wide range of biological processes, such as cell growth and proliferation, independently of changes in intracellular Na^+ and K^+. One of the first observations led to the discovery of the activation of p42/44 MAPK pathway and Ras protein in cardiomyocytes chronically treated with ouabain. However, it is now known that this pathway is activated from the close proximity of Na,K-ATPase to the epidermal growth factor receptor (EGFR), where ouabain binding to Na,K-ATPase induces EGFR transactivation and activation of Src tyrosine kinase. Then, adapter molecules are recruited to induce Ras activation, continuing the p42/44 MAPK cascade. In addition to modulating the action of the $Na^+/Ca2^+$ exchanger increasing the influx of $Ca2^+$, ouabain also induces $Ca2^+$ release from endoplasmic/ sarcoplasmic reticulum via interaction with the IP3 receptor, promoting the activation of transcription factors involved in events of cell proliferation, differentiation or even apoptosis [22-24].

In renal epithelial cells, 10 nM ouabain induced cell migration *via* secretion of metaloproteases, by activating focal adhesion kinases, Src and MAPKs.

On the other hand, high (toxic) concentrations of ouabain modulated cell adhesion, decreasing membrane expression of anchoring proteins like tight junctions, cadherins, desmosomes and the β subunit of Na,K-ATPase. These changes were followed by a sustained increase in tyrosine phosphorylation, MAPK activation, as well as an intense generation of reactive oxygen species (ROS) [25-27]. Corroborating this hypothesis, comparative studies in renal cell lines showed that reduced glutathione (GSH) was able to revert ouabain toxicity, decreasing both Ras expression and phosphotyrosine levels. Conversely, blockade of GSH synthesis was able to sensitize a cell line resistant to the toxic effects of ouabain, proving that at least part of its toxic effects resides in ROS induction [28].

However, other studies have demonstrated the activation of the stress protein p38 MAPK by ouabain, independently of the action of other family members (p42/44 MAPK and JNK) in different experimental models. In renal epithelial cells, this activation was induced by high concentrations of ouabain, correlating with the induction of cell death. In contrast, the decrease in the expression of the ABCC1 transporter occurred under physiological concentrations of ouabain (1 nM), suggesting a possible hormonal action in the regulation of the transport of several substances, including xenobiotics or even endogenous mediators [29].

2. Ouabain and cancer

Stenkvist and collaborators in 1982 had already found that, in patients who were not taking digitalis, the risk of recurrence of breast cancer within five years after mastectomy was 9.6 times higher than of that seen in patients who were taking digitalis. The authors suggested that cardiac glycosides have a modifying influence on the biologic aggressiveness of breast cancer [30]. These results were expanded and confirmed later by the same author [31], but not by a more recent meta-analysis performed by Osman *et al*. [32].

Nevertheless, according to Mijatovic and collaborators [33], epidemiological data along with reports based on *in vitro* and *in vivo* studies demonstrate cardiotonic steroid-mediated anti-cancer activity, and further emphasize the possibility of employing this class of compounds as anti-tumor agents.

Although the comprehension of the functions of cardiotonic steroids is still quite incomplete, the importance of this class of hormones seems to be considerable since endogenous cardiotonic steroids exhibit physiological functions that go far beyond regulation of sodium transport, as for example, regulation of cell growth and differentiation, apoptosis and proliferation. According to Bagrov and collaborators [7], expanding our understanding of this class of hormones will lead to novel and effective therapeutic strategies of great relevance to optimizing health and curing diseases.

Thus, the possible antitumor effect of cardiac glycosides such as ouabain has been investigated in cell lines of different types of cancer [34]. The effects on some cellular processes and signaling pathways were investigated and will be described as follows.

2.1. Cytotoxic effect of ouabain on tumor cells

Apoptotic cell death is believed to be the main mode-of-action of most conventional chemotherapeutic drugs, as well as radiation therapy, in tumor treatment [35, 36]. Taking this into consideration, the most pronounced effect produced by ouabain is the capacity of inducing apoptosis in different tumor cell lines at different concentrations [37-42].

Ihenetu and collaborators [37] exposed Jurkat cells, K-562 cells, and peripheral blood mononuclear cells (PBMCs) to digoxin (10–500 nmol/L) or ouabain (10–500 nmol/L) for 48 h. The authors verified that both digoxin and ouabain induced apoptosis in Jurkat cells but not in K562 cells or PBMCs. Treatment with digoxin significantly increased the percentage of apoptotic cells in a dose-dependent manner in Jurkat cells (IC50, 24 nmol/L), but not in K562 cells or PBMC cultures, compared to the untreated controls. Ouabain similarly induced apoptosis in Jurkat cells (IC50, 26 nmol/L) but not in K562 cells or PBMCs. Additional increases in the digoxin or ouabain concentration beyond the 500 nmol/L concentration did not significantly increase apoptosis in Jurkat cells.

In addition, the role of ouabain as an agent that induces the formation of reactive oxygen species (ROS) in tumor cells is well reported in the literature. Most of the studies showed alterations in the apoptotic pathways associated with changes in ROS or/and calcium levels. Yan and collaborators [39] showed that ouabain (0.3 and 0.5 μM) increased ROS generation in glioblastoma cells, suggesting that ouabain induced glioma cell apoptosis partly *via* ROS accumulation. This effect did not seem to be mediated by calcium overload, but by p66Shc

phosphorylation. According Ulivieri [43] active p66Shc could result in the increase in the levels of reactive oxygen species (ROS) in the intermembrane space of mitochondria T cells, leading to cristae remodelling and release of cytochrome-c. P66shc is an adaptor protein that belongs to the ShcA family. It mediates oxidative stress in many cell types and tissues [43]. Moreover, Yan and collaborators [39] showed also that Src/Ras/ERK signal pathway was involved in ouabain-induced p66Shc phosphorylation in glioblastoma cells.

Opening mitochondrial pore is a process regulated by calcium, and other factors like ROS [44]. Besides, ROS can act as an inductor of apoptosis. In the intrinsic pathway, ROS can act directly in the oxidation of the mitochondrial pores leading to cytochrome c release due to disruption of the mitochondrial membrane potential [45]. In the extrinsic pathway, ROS can act on death receptors [46].

The change in the levels of ROS and calcium, produced by ouabain, could, therefore, be associated with a series of pathways that induce cell death. Depending on the concentration and type of tumor cell, it is possible to observe a correlation between reactive oxygen species (ROS) and calcium overload. Xiao and collaborators [47] verified an increase in cytosolic levels of ROS and Ca^{2+} in OS-RC-2 (renal cancer) and NCI-H446 (small cell lung cancer) cell lines treated with 20, 40, 80, 160 and 320 nM of ouabain. They observed a decrease of proliferation rate in all cell lines in a time- and dose- dependent manner. An increase in the rate of apoptosis was also observed, though the cell lines analyzed presented different sensitivities to this glycoside.

Both extrinsic and intrinsic pathways of apoptosis could be activated by 10 μM ouabain, leading to the induction of apoptosis in DU145 human prostate cancer cells [42]. This study also showed an increase in ER-stress proteins like Grp78, ATF6β, p-PERKThr981, PERK, eIF2A, GADD153, CaMKIIβ, and caspase-4, indicating an activation of ROS-stimulated Ca^{2+} signaling pathway. However, ouabain could also activate other molecules belonging to the extrinsic apoptosis pathway, enhancing DR4, DR5, Fas, Fas Ligand, FADD and caspase-8. Moreover, the authors further observed that another effect caused by ouabain in DU145 cancer cells was associated with DNA damage.

Chou and collaborators [41] also described the activation of the extrinsic apoptosis pathway in an osteosarcoma cell line treated with 5 μM ouabain, demonstrating the increase of Fas, FADD and FasL caspase-9, caspase-3 and caspase-8 associated with intracellular Ca^{+2} release, even without modifying ROS production. In the same work, the authors demonstrated that mitochondrial dysfunction was correlated with increased expression of cytochrome-c, Afpa-1, PARP, AIF and also Endo G.

Additionally, ouabain was shown to increase the activity of pro-apoptotic proteins, like Bax, and also inhibit the activity of anti-apoptotic proteins [40-42]. For instance, OS-RC-2 cells treated with a range of ouabain concentrations demonstrated a dose-dependent increase in Bax protein levels and a decrease in Bcl-2 protein levels [47].

While the vast majority of studies indicate that ouabain induces cell death by apoptosis, some of them emphasized that ouabain can also induce autophagy. Autophagic cell death, also called type II programmed cell death (type II PCD), is a caspase-independent cell death program distinct from apoptosis. Autophagy is a tightly regulated catabolic process in which cells degrade their own components by enveloping them in double-membrane vesicles termed autophagosomes that are targeted for lysosomal degradation [48]. This process also plays a tumor-suppressing role and may at least partially contribute to the drugs' growth inhibition [38].

Regarding the correlation of this phenomenon and the action of cardiac glycosides, Meng and collaborators [40] also verified that ouabain treatment (25 nM, 50 nM or 100 nM) of Burkitt's lymphoma Raji cells increased the levels of procaspase-3 and cleaved-caspase-3. However, vacuole accumulation was also observed *via* transmission electron microscope (TEM) images of ouabain-treated Raji cells, indicating that these cells were undergoing autophagy instead of apoptosis. Thus, the authors concluded that both apoptotic and autophagic pathways were involved in ouabain-induced cell death. In their experiments, the expression of the autophagy-related proteins LC3-II and Beclin-1 was upregulated in ouabain-treated Raji cells. These proteins also were markedly induced by ouabain (25 nM) in human non-small lung cancer cells (A459 and H460 cell lines), however these cell lines also demonstrated up-regulation of Atg5 [38]. In this study, Wang and collaborators [38], observed that the AMP-activated protein kinase (AMPK) pathway was activated, resulting in mammalian target of rapamycin (mTOR) deactivation during autophagy induction. They concluded that

autophagy was induced and regulated by both mTOR and ERK1/2 pathways after the cardiac glycoside treatment.

Treating A549 cells with miR-34a and ouabain (30 nM) led to the observation of a large number of vacuoles, suggesting activation of autophagy-associated cell death [49]. These authors were studying the use of miRNA combined with small molecules in cancer treatment in order to reduce the effective dose needed and, consequently, adverse reactions. The authors used a high-throughput screen consisting of 10,000 small molecules of known biological activity to identify a possible combination therapy that could increase miR-34a therapeutic efficiency. The loss or reduced expression of miR-34a has been detected in a variety of tumors and cancer cell lines [50]. Interestingly, Rupaimoole and collaborators [49] identified ouabain as a top small molecule candidate to combine synergistically with miR-34a in killing lung cancer cells even at low doses. In fact, the role of ouabain in causing a synergistic decrease in viability with miR-34a treatment was confirmed by analysis in four lung cancer cell lines (A549, HCC827, H1975 and CALU6). Besides, a decrease in cell counts was also verified for pancreatic cancer (BXPC3 cell line) and colon cancer (HCT116 and HCT15 cell lines) after treatment with miR-34a and ouabain combined.

2.2. Controversial role of ouabain on cell migration

Another role investigated for ouabain is its influence on cell migration, one of the constituent steps of metastatic processes. However, unlike studies on ouabain mediated-apoptosis, these results are scarcer and perhaps, for this reason, more divergent.

Yang and collaborators [51] showed that ouabain suppresses the growth and migration abilities of glioma U-87MG cells through the inhibition of the Akt/mTOR signaling pathway and downregulating the expression of HIF-1α. In this study they found that, in this cell line, for a 24-h treatment, ouabain IC_{50} was 0.45 μM. A decrease in both cell viability and motility was observed and the authors suggested that these responses were associated with decreased phosphorylated levels of AKT and mTOR protein, accompanied by a decrease in HIF1a protein levels.

Shih and collaborators [52] investigated the ouabain antimetastatic effect on human osteosarcoma U-2 OS cells. Results indicated that ouabain significantly decreased the percentage of viable cells at 2.5-5.0 μM, therefore, smaller concentrations (0.25-1.0 μM) were selected for migration inhibition studies. Ouabain inhibited cell migration, invasion and the enzymatic activities of MMP-2 metalloproteinase, as well as its gene and protein expression. Therefore, the authors suggested that ouabain may be a candidate for developing preventive agents against human osteosarcoma cancer metastasis.

Another group of experiments were performed aiming the comprehension of the effects of physiological levels of ouabain. Ruanghirun and collaborators [53] analyzed whether picomolar concentrations of this glycoside could interfere in the cell detachment of the human lung cancer cell H23. They observed that concentrations of 0-10 pM were not cytotoxic nor affected cell proliferation. Only the concentration of 50 pM was capable to evoke cytotoxic effects decreasing in 30% of the number of viable cells and to induce apoptosis. However, sub-toxic concentrations (ranging from 1 pM to 10 pM) reduced the number of adherent cells in a time- and dose-dependent manner. They concluded that physiologic concentrations of ouabain can impair weakened cancer cell adhesion to the extracellular matrix (ECM) and facilitate detachment. Furthermore, in cells exposed to this cardiac glycoside the activation of the focal adhesion kinase (FAK) and ATP-dependent tyrosine kinase (Akt) pathway and the reduction in the levels of integrin proteins by lysosomal degradation were observed.

Pongrakhananon *et al.* [54] have revealed that treatment with ouabain at physiological concentrations up to 30 pM, was able to inhibit the migratory and invasive activities of the human non-small cell lung cancer (NSCLC) cell line H292. According to the authors, the effects of ouabain on cell migration are associated with decreased activation of FAK and Akt and expression of cell division cycle 42 (Cdc42). Furthermore, ouabain was shown to inhibit tumor growth in spheroids and decrease cancer cell adhesion to endothelial cells. However, the compound had no significant effect on anoikis of the cells. In the referred study, once again the ROS role on ouabain effects was checked. The addition of ROS scavengers (N-acetylcysteine and glutathione) could reverse the effect of ouabain on cell migration. The authors also tested whether ouabain was able to inhibit migration in H460 cells, another NSCLC cell line. The results showed that, as well as for H292 cells, treatment of the H460 cells with ouabain at the 0–30 pM concentration range significantly attenuated cell migration in a

dose-dependent manner, as well as a decrease in activation of Akt and FAK.

Ninsontia and Chanvorachote [55] exposed human lung cancer H460 cells to physiological concentrations of ouabain (up to 40 pM). The results of the proliferation assay indicated that 10 and 20 pM ouabain significantly inhibited cell proliferation while the concentration of 40 pM induced apoptosis. Ouabain (20 pM) caused the reduction of integrins α4, α5, αv, β3 and β4, whereas it had no effect on integrins β1 and β5. As expression of integrins α5, αv and β3 was shown to enhance the metastatic potential of lung cancer, the suppression of such integrins mediated by ouabain may be responsible for its anti-metastatic activities. According to the switch patterns of integrins, ouabain treatment resulted in a dramatic reduction of cell colony size and inhibition of cancer cell migration. However, ouabain-induced integrin switch had only a slight effect on chemotherapeutic drug susceptibility including cisplatin, etoposide, paclitaxel and doxorubicin. These results suggest that ouabain may have no effect on chemotherapeutic resistance in these cells since it had no effect on integrin β1 but it may have a role in suppressing cancer metastasis *via* integrin regulation.

2.3. Ouabain action on the MDR phenotype

When evaluating the effectiveness of a possible anti-tumor drug it is necessary to consider one of the biggest obstacles in the treatment of cancer: the multidrug resistant (MDR) phenotype. This phenotype is characterized by a set of mechanisms such as: changes in the regulation of genes that involve apoptosis, increased drug detoxification and the activation or overexpression of drug-exporting proteins [56, 57]. One of the main mechanisms is the overexpression of ATP-binding cassette (ABC) transporters, including MDR1 (ABCB1), MRP1 (ABCC1) and BCRP or MXR (ABCG2) [58].

Studies were performed by different groups using different cell lines addressing the possibility that ouabain might modulate this phenotype. Brouillard and collaborators [59], using lung cancer cells (Calu-3) treated with 0.1, 0.2 and 0.5 μM ouabain for 24 h, observed an increase in *MDR-1* mRNA when compared with the control. The same was also observed in human colon cell lines T-84 and HT-29 and hepatic HuH7 cells, but not in HeLa cells. However, this increase was reversible after 24 hours culture in the absence of ouabain. In Calu-3 cells, this increase in MDR-1 gene expression was accompanied by an increased synthesis of P-glycoprotein (P-gp). The authors attributed these results to a change in the levels of cytosolic ions associated with an inhibition of Na,K-ATPase, since the concentrations used in this study were very close to that responsible for the inhibition of this pump.

However, the opposite has also been reported. Ouabain was capable of reversing the MDR phenotype in EC109/CDDP cells [60] *via* the inhibition of the canonical Wnt/β-catenin signaling pathway. The authors exposed resistant (EC109/CDDP) and sensitive (EC109) cell lines to a wide range of ouabain concentrations (0.001, 0.01, 0.1, 1, 10, 100, 1000 nM) for 24 h and observed that this cardiac glycoside was very effective against both drug-sensitive and MDR cancer cells. Their results revealed that 20 nM ouabain after 24 hours may potently downregulate P-gp protein and gene expression, contributing to its MDR reversal activity. The authors suggested that ouabain interferes with the Wnt/β-catenin signaling pathway, as this pathway is closely associated to P-gp expression [61]. This glycoside efficiently inhibited the transfer of Wnt/β-catenin into the nucleus in EC109/CDDP cells. However, ouabain's ability to reverse the resistance phenotype may not be solely linked to ABC transporters. Alterations on pathways evoked by ouabain could be linked with its association with Na,K- ATPase. Mijatovic and co-workers [62] strongly suggest that Na,K- ATPase could represent a novel target to treat malignancies. This pump could also be the Achilles heel of the MDR phenotype [63]. Therefore, ouabain, as well as other cardiac glycosides, has aroused the interest in cancer treatment due to their high affinity for Na/K- ATPase. Saeed and collaborators [64] measured the cytotoxic activity of ouabain in drug-sensitive parental CCRF-CEM leukemia cells and their multidrug-resistant P glycoprotein-overexpressing subline, CEM/ADR5000. According to the authors, the correlation analysis for ouabain did not reveal any statistically significant associations between cellular responsiveness to this cardiotonic glycoside and ABCB1 expression and function, indicating that multidrug-resistant cells may still respond to ouabain. Besides, the authors observed neither a direct nor inverse correlation with MRP1/ABCC1 expression, which does not indicate a role of ouabain as substrate of MRP1/ABCC1.

The literature data are contradictory concerning MRP1 and ouabain. Cells with acquired ouabain

resistance overexpressed ABCC1 [65]. On the other hand, ouabain has been reported to downregulate ABCC1 expression [29].

2.4. Cell metabolism and ouabain

Dysregulated metabolism is one of the hallmarks in cancer [66]. There is evidence that reveals that ouabain impairs cell metabolism. AMPK is an energy sensor that acts in the maintenance of the homeostasis of the cell energy in response to a metabolic stress [67]. Shen and collaborators [68] observed that ouabain could activate both AMPK and SRC pathways in A549 lung cancer and MCF7 breast cancer cells following treatment for 6 hours with 25 nM ouabain. This activation was sustained for 48 hours, but their data suggest that AMPK activation acts upstream of the Src signaling pathway in these cancer cells. AMPK activation by ouabain, leads to a marked decrease in intracellular ATP levels in a time-dependent manner and also impairs mitochondrial respiration in A549 and MCF7. In the A549 cell line this compound was capable to decrease glycolysis with a reduction in lactate production and glucose uptake. However, in MCF7 a less pronounced effect was observed. This finding indicates that the AMPK activating-agent-induced metabolic response may vary depending on the cancer cell type.

AMPK negatively regulates glycolysis by inhibiting HIF-1α, a key regulator of glycolysis [69]. Studies have shown that 50 nM ouabain is able to inhibit the HIF-1α and HIF-2α in Hep3B cell line (hepatocellular carcinoma) [70]. Yang and collaborators [51] also reported a decrease in HIF-1α protein levels, though at higher concentrations (2.5 and 25 μM ouabain), in glioma U-87MG cells.

HIF also is involved in both intrinsic and extrinsic activation of tumor-associated inflammatory signaling [71, 72]. A large body of evidence suggests that NF-κB and HIF-1 link inflammatory signaling to hypoxia [73]. In response to inflammation, inhibitory IκB proteins are dissociated from NF-κB allowing its nuclear translocation and activation of tumor-promoting genes including interleukin-6 (IL-6), cyclooxygenase 2 (COX-2) [73, 74]. The result of Saito and collaborators [75] demonstrates the anti-tumoral capacity of ouabain in oral squamous carcinoma cells. This type of tumor may have its progression associated with spontaneous secretion of cytokines such as IL-8 and IL-1α. Thus, the authors observed that 30 μM ouabain is capable of generating different effects on cytokines, since while IL-8 was diminished, IL-1α was increased. The authors justify the decrease in IL-8 by ouabain's ability to act on NF-κB. According to the results obtained, ouabain inactivates the upper subunit of NF-κB, in addition to preventing the translocation of this transcription factor to the nucleus. In contrast, the activity of the transcription factor AP-1, which would act on the transcription of IL-1α, was not altered in the presence of ouabain, and the work still reveals that C-fos and C-jun are even more expressed in the presence of ouabain.

3. Inflammation, cancer and ouabain

Besides being considered a physiological response, non-resolving inflammation is an undesirable process since it is associated with many pathological states [76]. Persistent inflammatory condition is an indispensable participant in the neoplastic process development [77]. Inside the tumor microenvironment, many mediators, e.g., proinflammatory cytokines, complement proteins [78-80] and inflammatory cells, such as tumor-associated macrophages (TAMs) [81] and neutrophils [82], contribute to tumor growth and further progression [71, 83]. Several studies, both *in vivo* and *in vitro*, are under way to better understand the importance of inflammation on cancer development and cancer therapy [84].

3.1. Ouabain and the inflammatory process

Inflammation is a physiological response, coordinated by the immune system, which can eliminate pathogens and promote tissue repair. This process is regulated by several signaling pathways and requires the interaction of different cells types, such as leukocytes [85].

Depending on the intensity and extent of the inflammatory response, including whether it is systemic or local, metabolic and neuroendocrine changes may occur that trigger immune system activation [86]. Different endogenous (e.g., microbiota and epigenetic alterations) and exogenous (e.g., quality of sleep, pollution, and diet) factors can contribute to acute inflammatory processes leading to chronic systemic inflammation, with possible break of immune tolerance and tissue changes [86-88].

The relationship between ouabain and inflammation was initially observed by Lancaster and Vegad [89]. They demonstrated that tupertin-induced vascular permeability in the skin and the pleural cavity of sheep was suppressed by ouabain [89]. Later,

preliminary works showed that ouabain could modulate leukocyte migration [90, 91]. During several years, the relationship between ouabain and the immune system was being reported [92, 93], including a well-established ability to modulate inflammation [94].

Using classical murine models of inflammation, De Vasconcelos and coworkers [95] presented an established ouabain anti-inflammatory property at low doses. In this work, ouabain was able to reduce paw edema induced by different phlogistic agents (i.e., carrageenan, compound 48/80, zymosan, prostaglandin E_2, and bradykinin). This ouabain effect could be due to its properties of inhibiting histamine release [96] and reducing vascular permeability [97]. Additionally, ouabain displayed an antinociceptive effect against the inflammatory model of pain [95].

Different studies have been demonstrating the effect of ouabain on inflammatory processes induced by different microbial phlogistic agents. In a model of peritoneal inflammation induced by zymosan in mice, a fungal wall polysaccharide, ouabain reduced levels of cytokines TNF-α and IL-1β, without interfering with levels of IL-6 and IL-10. This effect was associated with a reduction in the translocation of the nuclear factor κB (NF-κB) to the nucleus [97]. Furthermore, it was shown that ouabain does not modulate the production of nitric oxide (NO) in the culture of peritoneal macrophages of Swiss mice stimulated by bacterial lipopolysaccharide (LPS) [97], differently from what Sowa and Przewłocki [98] reported in peritoneal macrophages of rats, in which ouabain increased NO levels induced by LPS. Also, in rats, some reports revealed that ouabain increased histamine secretion induced by different agents on mast cells [99, 100].

It has also been shown that ouabain reduces the inflammatory response triggered by infection caused by *Leishmania amazonensis* in mice [101], reducing TNF-α and IFN-γ levels. Additionally, in a sepsis model, Dan and collaborators [102] showed that ouabain reverses the sepsis immunoparalysis, increasing levels of TNF-α, IFN-γ, and GM-CSF, and thus promoting animal survival. It also has been reported that ouabain attenuates sepsis by modulating TLR signaling and apoptosis of T cell [103].

Leukocyte migration, a key feature of inflammation, is modulated by ouabain, mainly neutrophil chemotaxis. This steroid was able to reduce mice neutrophil migration induced by zymosan [97], *L. amazonensis* [101], ovalbumin [104], and LPS [105]. Other studies have also demonstrated the inhibitory effect of ouabain on rabbit [91] and human neutrophil chemotaxis [106]. Little is known about the mechanisms involved in this effect, but could be related to CD18 expression reduction, an adhesion molecule [107], or even inhibiting chemokine receptor recycling [106]. Moreover, in old mice, ouabain reduces immune cell infiltration in liver [108].

Some reports have documented ouabain effects on pulmonary inflammation. Ouabain negatively modulates the pulmonary allergic inflammatory process, by reducing eosinophilic migration, Th2 profile cytokines (e.g., IL-4 and IL-13), and mucus production in bronchioles [104]. Furthermore, ouabain controls acute lung injury induced by LPS, possibly by inhibiting lung NF-κB and MAPK signaling pathways [105]. Also in the pulmonary microenvironment, ouabain was shown to reduce lung fibrosis [109], which is a feature of some respiratory inflammatory diseases, such as asthma [110]. This anti-fibrotic effect of ouabain could be related to the downregulation of TGF-β/TGF-βR2 axis [111, 112].

On the other hand, different studies revealed an ouabain pro-inflammatory effect, triggering acute lung inflammation with increased migration of neutrophils to lung tissue and increased production of mediators such as leukotriene B4 and PGE_2, in addition to cytokines such as IL-6, TNF-α, and IL-1β [113, 114]. Additionally, Chen and co-workers [115] reported that ouabain leads to peritoneal inflammation and macrophage inflammatory response through NF-κB activation and cytokine production.

The apparent duality of ouabain effects on inflammation can occur due to different sensitivities of the animal species studied (BALB/c, Swiss, or C57BL/6 mice; rats; humans), administration route used, presence of a previous inflammatory stimulus, or even different concentrations of this glycoside. Indeed, high ouabain levels could trigger an immune system activation, possibly by increasing reactive oxygen species, and promote a pathological inflammatory response [116]. It has been shown that high doses of ouabain can induce the production of IL-1β *via* activation of NLRP3 inflammasome in macrophages and cardiac tissue, contrasting with inhibitory effects of ouabain on the production of this cytokine demonstrated by Leite *et al*. [97] in low doses.

The relationship between ouabain and inflammation has also been documented [117]. In a model of neuroinflammation stimulated by LPS, it was observed that ouabain in low concentrations demonstrated anti-inflammatory activity by reducing the p65 subunit of NF-κB. In addition, mRNA levels of inducible nitric oxide synthase (iNOS) and the proinflammatory cytokine IL-1β were also reduced by this glycoside [118, 119]. In contrast, micromolar concentrations of this steroid induced activation of NF-κB and an increase in pro-inflammatory cytokines (TNF-α and IL-1β), together with an increase in the cyclic adenosine monophosphate responsive element binding (CREB) in rats' primary cerebellar neuronal culture and direct administration of ouabain in the rat hippocampus [120-122]. In addition, a study using glial cells, which can regulate inflammation and brain damage, showed that treatment with ouabain reduces the activation of NF-κB after stimulation with LPS [123].

3.2. Ouabain and tumor microenviroment

The inflammatory process might promote tumorigenesis and, therefore, prolonged inflammation should be avoided. However, it also determines cellular infiltration in the tumor microenvironment, the subsequent immune response and cancer fate [84]. The cancer microenvironment is composed of tumor cells, stromal cells, tissue-resident macrophages and infiltrating cells from the immune system. The balance of the different cells and stimuli may inhibit or restrain cancer growth, but it also may lead to tumor progression. But, it is necessary to take into account that immune cells infiltrating the tumor site also suffer the influence of the environment.

Human monocyte-derived macrophages originate in the bone marrow. As monocytes they have a very short life span in the circulation, of approximately 1 to 3 days followed by migration into target tissues or organs where they undergo activation and differentiation. Monocyte differentiation depends on the composition of the surrounding environment giving rise to M1 or M2 macrophages, that are distinct both phenotypically and functionally. Cytokines such as IFNγ may induce monocyte differentiation into M1 cells. This kind of macrophage is considered pro-inflammatory and produce IL-1β, IL-6, IL-12, IL-23, and TNF-α. Differentiation into M2 macrophages may be induced by IL-4 and IL-13 and they characteristically produce IL-10 and TGF-beta, having an important role in the healing process [124]. M1 macrophages are the typical antigen-presenting cells, expressing both MHC class II and B7 molecules (CD80 and CD86) for the stimulation of T cells, whereas M2 macrophages lack those receptors but express CD16 [125].

The possibility of re-programming tumor-associated macrophages and, in this way, modulating the immune response against the tumor has been proposed [126].

Monocytes express on their membranes the pattern recognition receptor CD14 that detects microbial products such as lipopolysaccharide leading to innate and adaptive immunity. The expression of this receptor increases during monocyte differentiation into macrophages. Using a concentration of 100 nM ouabain a significant reduction in CD14 expression by human monocytes was observed. This was not a result of cell death nor receptor endocytosis, and proved to be reversible. The use of pharmacological inhibitors demonstrated that CD14 reduction was due to EGFR transactivation and the activation of the p38 MAPK protein. Accordingly, the levels of P-p38 were increased on monocytes after ouabain treatment. Furthermore, these effects were not related to Na,K-ATPAse endocytosis. At this concentration, ouabain also inhibited a subpopulation of monocytes considered pro-inflammatory, CD14 + CD16 + [125, 127]. Surface activation markers such as CD69, HLA-DR, CD86, and CD80 were also increased by ouabain as well as intracellular calcium levels. Some functions such as phagocytosis were increased *whereas ouabain-treated monocytes failed to stimulate lymphocytes in a mixed reaction.* Furthermore, ouabain-treated macrophages presented a mixed cytokine profile producing IL-1 β and TNF-α, as well as IL-10 and VEGF [128].

Dendritic cells are also present in the tumor microenvironment. These cells derive from bone marrow myeloid lineage and in humans represent only 0.1–0.5% of mononuclear cells present in peripheral blood. This cell type circulates in the blood as an immature precursor prior to migration into peripheral tissues. Dendritic cells exhibit a large spectrum of phenotypes and activities, being extremely sensitive to microenvironment signals, such as tumor-derived cytokines [129, 130]. In the tumor microenvironment the tolerogenic pathway is increased in relation to the effector pathway [131].

During differentiation of monocytes into dendritic cells, a number of alterations regarding surface

proteins are observed. A feature of this process is the loss of the expression of the surface molecule CD14 by monocytes and the acquired expression of molecules of the CD1 family. Despite the fact that ouabain leads to the loss of CD14 it does not reflect a shift in differentiation into dendritic cells because it failed to induce CD1a expression [127].

When differentiated and activated, dendritic cells increase the expression of costimulatory molecules, such as CD80, CD86, and CD40, and express high levels of CD83 and MHC class II on their surfaces [131]. Using cells obtained from human peripheral blood, the role of ouabain was investigated *in vitro*. In this condition, immature dendritic cells were obtained from culturing human monocytes with IL-4 and GM-CSF for 5 days, and mature dendritic cells by further differentiation and stimulation of these immature cells for 48 h with TNF-alpha. A concentration of 100 nM ouabain abolished TNF-induced CD83 expression and IL-12 production by these cells, whereas CD80 expression was unaffected. Furthermore, surprisingly, ouabain increased HLA-DR and CD86 expression and did not affect dendritic cells' allostimulatory capacity [132]. Dendritic cells activated in the presence of oubain mature into a different category and its impact on tumor growth is unknown.

On the other hand, cardiac glycosides have been identified as immunogenic cell death (ICD) inducers [133]. The importance of ICD is that it leads to the transition of dendritic cells from the immature into the mature phenotype, as well as stimulating these cells to activate T lymphocytes [134].

3.3. Ouabain and lymphocytes

By interfering with the inflammatory process as well as with antigen presenting cells, it should be expected that ouabain would have an effect on the immune response. Studies *in vitro* and *in vivo* have shown that lymphocytes may also be directly affected by ouabain. Human peripheral lymphocytes present over 40 000 ouabain-binding sites per cell [135] and a number of studies have shown that lymphocyte proliferation induced by a variety of stimuli may be inhibited by ouabain [136-146]. Most studies described that the failure to progress through the cell cycle resulted from the lack of expression of CD25 and/or the lack of IL-2 secretion. It is still not clear which signalling pathways are involved and which other mechanisms are responsible for this lack of proliferation, but as seen above in the various studies using tumor cells, this inhibition of proliferation is probably a more general phenomenon.

It was not just inhibition of proliferation that resulted from lymphocyte stimulation in the presence of ouabain. Activation of the apoptotic process by ouabain was also observed [146]. Furthermore, when mitogen-activated lymphocytes were exposed to 100 nM ouabain they became more sensitive to the CD95 activation pathway [147]. This is similar to what had been reported using Jurkat cells [148-150]. Furthermore, a synergy between ouabain and PHA resulting in plasma membrane depolarization and dissipation of the mitochondrial membrane potential has been observed and might be responsible for that increased susceptibility [147].

Another cell type that plays an important role in cancer development is the natural killer (NK) cell population. Ouabain did not inhibit the cytotoxic activity of these cells [142] nor that of lymphokine-activated killer (LAK) cells, but despite not affecting their cytotoxic capacity it inhibited the activation phase of LAK-cells [143]. Conversely, ouabain may even enhance target cell death as it has been observed that cytotoxicity produced by NK-derived perforin is calcium dependent [151].

When animal models were used to study the effect of ouabain on cells from the immune system, it has been described that mouse bone marrow cells exposed to ouabain increased the number of erythroid progenitor cells and that there was a decrease in the formation of pluripotent stem cells and granulocyte progenitor cells [152, 153].

Furthermore, mice treated *in vivo* for three consecutive days with ouabain displayed different responses depending on the tissue under study. An increased percentage of myeloid cells with a decrease in the mature B cell population was observed in the bone marrow. In the spleen and peripheral blood the number of mature B cells was also diminished. The most affected subpopulation was folicular B cells, but a reduction in the number of marginal zone B lymphocytes was also observed. This was accompained by an increase in B cells in mesenteric lymph nodes. However, IgM and IgG levels in the serum were unaffected [154, 155]. Similar to what was observed with B cells, there was a reduction in the numbers of T cells, mainly CD4 lymphocytes and especially regulatory T regs. On the other hand, contrary to what had been

observed with B cells there was no accumulation in the mesenteric lymph nodes. Secretion of IL-2 by T cells was decreased, as had been reported in other studies, and this might explain the effect on T regs since this cytokine is involved in the peripheral conversion and maintenance of these cells [156].

In very young mice, ouabain given once did not directly affect T cell precursors in the thymus, however, it synergized with glucocorticoids in the induction of thymic atrophy and the increased apoptosis of double positive thymocytes [157]. Similarly, despite not being able to induce membrane depolarization in thymocytes ouabain augmented depolarization glucocorticoid-induced. Murine lymphocytes obtained from lymph nodes, spleen and thymus of adult animals did not suffer membrane depolarization when exposed to 100 nM of ouabain, a concentration capable of affecting the activity of these cells [93]. Different signaling pathways were also explored and it was observed that concentrations as low as 10 nM, decreased the levels of phosphorylated p38 induced by Con A [158].

Ouabain may be considered an immunomodulatory molecule and many of its actions seem to be independent of the inhibition of the Na, K pump. Notwithstanding, it is still not clear whether ouabain would be capable of modulating the immune response to tumor growth *in vivo*.

4. Conclusion

Taken together, all the physiological changes modulated by ouabain suggest that in addition to its actions in different inflammatory processes, ouabain also acts on tumor development, suggesting a new mode of action for this substance.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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