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Commentary

UNBS1450 from *Calotropis procera* as a regulator of signaling pathways involved in proliferation and cell death

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ABSTRACT

Despite significant progress in oncology therapeutics in the last decades, the urge to discover and to develop new, alternative or synergistic anti-cancer agents still remains. For centuries it has been known that the coarse shrub *Calotropis procera* is a very promising source of ascaricidal, schizonticidal, anti-bacterial, anthelmintic, insecticidal, anti-inflammatory, anti-diarrhoeal, larvicidal and cytotoxic chemicals. Different compounds like norditerpenic esters, organic carbonates, the cysteine protease procerain, alkaloids, flavonoids, sterols as well as numerous types of cardenolides have provided this plant for centuries with scientists' interest. The chemical class of cardenolides and their related bioactivity evaluation and structure–activity relationship (SAR) studies pointed out their therapeutic utility and led to the discovery of promising drug candidates. Recently the cardiotonic steroid UNBS1450 **01** (derived from 2-oxovoruscharin **02**) from *C. procera* was shown to additionally exert an anti-cancer activity. UNBS1450 **01** has been proven to be a potent sodium pump inhibitor, showing anti-proliferative and cell death-inducing activities. This anti-cancer potential of UNBS1450 **01** is achieved by disorganization of the actin cytoskeleton after binding to the sodium pump at the cellular membrane, by inducing autophagy-related cell death, by repressing NF-κB activation as well as by down-regulating c-Myc in cancer cells. We aim to review pharmacologically important chemical extracts from *C. procera* and focus more specifically on the anti-cancer activities of UNBS1450 **01**.

1. *Calotropis procera* as a source of natural drugs

1.1. C. procera

According to literature, the origin of synthetic drugs development lies within the upcoming of The Industrial Revolution. The potentially more active synthetic products and the increasing economic power of pharmaceutical companies were the main reasons for a preference for these new products compared to the natural compounds from mineral, plant and animal sources used for ages.

However, a significant number of these synthetic drugs are obtained from natural precursors; it is even estimated that 11% of the drugs considered as basic and essential by the World Health Organisation (WHO) are strictly of plant origin. Digoxin, quinine, quinidine, vincristine, vinblastine, atropine, morphine and codeine might be the most prominent. Furthermore, according to Yue-Zhong Shu, 60% of current or future anti-tumor and anti-infectious drugs are of natural origin [1].

C. procera, a member of the Asclepiadaceae, is a woody, broadleaf evergreen coarse shrub, 3–5 m tall, widely growing in the tropics. It is distributed in arid to semi-arid regions of the Caribbean, Central America, South America, Africa, India and Israel, mainly appearing on plains and in the uplands. For decades, *C. procera*, also referred to as "The Apple of Sodom" has been used especially in traditional folk medicine because of its pharmacologically active compounds found in its roots, bark, leaves and mainly in its latex which exudates from damaged leaves. Meanwhile, chemical extracts from *C. procera* have been shown to have ascaricidal, schizonticidal, anti-bacterial, anthelmintic, insecticidal, anti-inflammatory, anti-diarrhoeal, larvicidal, cytotoxic and analgesic effects, thus explaining a growing demand in today's medical research for the different parts of the plant [2–5].

1.2. The latex of C. procera

More than 12,000 plant species contain latex, a milky fluid in which occurs a wide range of proteins, and for some 1000 species also *cis*-polyisoprene that is the polymeric hydrocarbon of rubber [6]. As the latex is abundant in the green parts of the plant, it is thought to be produced and accumulated as a defense strategy against viruses, fungi and insects. Indeed, besides proteins

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involved in rubber biosynthesis, latex fluids have been shown to contain proteins implicated in plant defense and oxidative metabolism [7,8]. Today, latex is considered as a promising source of pharmacologically active molecules that might be chemically modified to improve their effectiveness.

With regards to *C. procera*, although the pharmacological potential of its latex has well been proven, only few active latex molecules have been identified until today; most often latex is either used as an entity or is only poorly processed, mainly in order to eliminate rubber compounds. Usually, the crude latex from *C. procera* is collected from non-cultivated plants by small incisions near the younger leafs and is mixed 1:1 (v/v) into distilled water. The material is then kept under constant, gentle, agitation in order to prevent it from precipitating. It is maintained at room temperature until being centrifuged at 5000 × *g*, 10 min at 4 °C. The precipitated material is pooled apart while the supernatant is further dialysed using membranes of 8000 Da cut-off, thus keeping the protein fraction, before being lyophilized. Generally, authors use both, the dialysis liquid containing low molecular weight compounds, called the dialysis latex (DL), and the non-dialyzable latex (NDL) obtained after exhaustive dialysis and containing concentrated proteins.

Soares et al. were among the first to show benefic, anti-nociceptive effects of fractionated latex (NDL) [9]. After acetic acid induced writhes in mice, the latex was capable of inhibiting up to 100% the abdominal constrictions in a dose-dependant manner (12.5–50 mg/kg). Morphine was used as a positive control (54.5% of inhibition). Similar results were obtained for formalin-induced paw licking for a hot plate test. In contrast to morphine, they found that the opioid antagonist nalaxone did not alter the latex-induced antinociceptive effect.

In 2006, Freitas et al., went a bit further and discovered pro- and anti-inflammatory potentials of dialysed and non-dialysable latex [10]; dialysed latex of *C. procera* was shown to increase induced leukocyte, especially neutrophil, migration whereas anti-inflammatory and anti-nociceptive activities were associated to non-dialysable latex.

Since *C. procera* has been known to be effective against several human diseases, the aim of the work of Ramos et al., was to elucidate larvicidal activities of different fractions of the plant's latex [11]. Their evaluation was based on egg hatching and larval development of *Aedes aegypti*. A 10 mg/mL concentration of NDL was able to completely block egg hatching; the inhibiting potential of the DL fraction was significantly lower as a 5 times greater concentration (50 mg/mL) was needed in order to achieve 0% of egg hatching. Both, NDL as well as DL, also triggered severe larvae mortality in a dose and exposure time-dependent manner. Chromatography analysis revealed different protein and amino acids of NDL and DL latex, thus delivering promising outlooks for future preparations of *C. procera* latex as a larvicidal compound.

In contrast to latex fractionation, some authors used air dried latex; in most examples, latex was extracted from the aerial parts of the plant before being dried under shade (DL, dried latex).

After being triturated 1:1 with gum acacia, DL administrated orally to albino rats and guinea pigs inhibited carrageenan and Freund's adjuvant-induced edema as well as granuloma formation. Further anti-inflammatory activities of DL resulted in fluid exudation inhibition and in reduced UV-induced erythema onset [12]. Similar results were obtained by Arya and Kumar. Shade dried DL, soxhlated to get methanolic extracts and triturated with gum acacia as seen before, showed anti-inflammatory activity against histamine, serotonin, compound 48/80, bradykinin and prostaglandin mediated inflammatory reactions in induced rat paw edema [13]. The same research team also demonstrated that DL, triturated this time in normal saline, is able to play an antioxidant and anti-hyperglycemic role in alloxan-induced diabetes in rats [14]. Contradictory to these findings, there is some evidence that DL is also capable of inducing pro-inflammatory effects. In 2003, Shivkar et al. proved DL to be able to produce dose-dependent inflammatory responses resulting in rat paw edema similar to carrageenan. Further studies suggested biogenic amines, especially histamine, to be involved in DL driven inflammation [15].

Similarly, by extracting DL sequentially with petroleum ether and methanol followed by a silica gel chromatography fractionation, Choedon et al. applied a DL treatment on X15-*myc* transgenic, hepatocellular carcinoma developing mice [16]. Not only did they discover an in vivo chemopreventive effect of DL, they also noticed that DL has cytotoxic effects on Huh-7 and COS-1 cancer cell lines. By chromatography separation, they isolated a polar fraction responsible for the observed cytotoxic potential. Apoptosis analysis revealed induced DNA fragmentation as a mechanism.

1.3. Ethanolic, methanolic and aqueous extracts

Phytochemically, the plant has been investigated since the 1960s, especially for cardenolides [17,18], triterpenoids [19,20], anthocyanins [21] and hydrocarbons [22]. Besides the use of plant latex, aqueous, methanolic, ethanolic and other organic plant extracts from different parts of the plant, namely flowers, buds, roots, stems and leaves, were among the very first approaches to develop natural drugs for classical and alternative medicine. In 1979, Malik and Chughtai described antimicrobial activity against pathogenic bacteria [23]. Moreover, organic extracts of *C. procera* were also shown to possess nematocidal [24,25], larvicidal [26], anti-fertility [27] and even anti-cancer potentials [28,29]. In 1987, Mascolo et al. further elucidated the biological properties of *C. procera* and, using ruminant animal models, discovered anti-inflammatory, analgesic, antimicrobial and antipyretic activities in extracts from *C. procera* flowers. In the late 1980s, Fenado et al. established a standard method to get crude aqueous extracts (CAE) from powdered *C. procera* flowers [30]. Similarly, in 1994 Asuzu and Onu managed to get crude methanolic extracts (CME) from *C. procera* using a Soxhlet apparatus [31]. Others used filtered ethanolic extracts to elucidate the plant's pharmacological potential [32,33].

Meanwhile, the ethanolic extracts of flowers, buds, roots, stems and leaves were discovered to have a schizontocidal potential *in vitro*. The different fractions of *C. procera* showed dose-dependant inhibitory effects on chloroquine sensitive and resistant *Plasmodium falciparum* strains. Antimalarial screening had been performed according to the method of Ang et al. [34]; parasitaemia had been adjusted to 1–1.5% before different concentrations of *C. procera* fractions were added. After an incubation time of 36 h, each sample was analysed under the microscope.

Furthermore, both, aqueous and methanolic extracts of powdered *C. procera* flowers, were shown to possess time-dependent anthelmintic activities *in vitro* [35]. Based on nematode egg counting in sheep feces, the authors confirmed their observed results *in vitro*: a maximum of 88.4% egg reduction (ECR, egg count percent reduction) had been recorded in sheep treated with CAE and 77.8% in sheep treated with crude powder. CME had been proven to be less effective.

Besides further insecticidal properties [36], organic *C. procera* extracts also possess interesting cytotoxic effects. Compared to the reference compound cisplatin (IC_{50} 0.9 μg/mL), Smit et al. demonstrated that ethanolic flos extracts (IC_{50} 1.4 μg/mL) have potent growth inhibition capacities when used on COLO 320 tumor cells.

More specific spectral data analysis of organic extracts of the plant revealed the presence of a norditerpenyl ester, calotropterpenyl, two pentacyclic triterpenoids, calotropursenyl acetate and

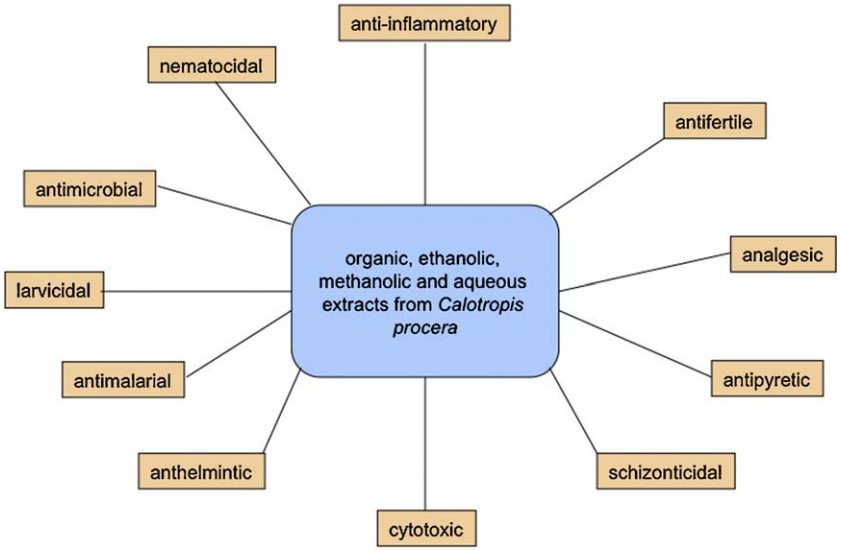

Fig. 1. Illustrative representation of the different pharmacologically important potentials of *Calotropis procera* extracts.

calotropfriedelenyl acetate as well as an organic carbonate, namely 2-propenyl-2′-hydroxyethyl carbonate [37,38] (Fig. 1).

1.4. Procerain

By further method standardizations and working condition optimizations, the next step in characterizing the plant's pharmaceutical potentials consisted in the isolation of new, specific and pharmacologically important proteins and compounds, especially with an enzymatic activity.

By default, plants are usually rich sources of various kinds of proteases, enabling protection, development of the plant, ripening of its fruits [39], nutritional reserve, degradation of seed storage proteins thus allowing germination [40], activation of proenzymes as well as elimination of defective proteins [41]. Proteolytic enzymes from plant sources, are of interest for the pharmaceutical research as the have been proven to be active over wide ranges of temperature and pH [42]. Consequently, an important number of proteases have been isolated from lattices, fruits and seeds. Among these, most belong to the cysteine super family, one of five catalytic types of proteases [43]. Concerning the different *Calotropis* species, since 1979 it has been known that the latex of *Calotropis gigantea* contains four cysteine proteases, namely Calotropin FI, FII, DI and DII [44]. More recently, Dubey et al. purified a new cysteine protease from *C. procera*, named procerain.

Procerain was isolated by applying a 50% ammonium fractionation of crude, gumless latex followed by CM-sepharose and SP-sepharose cation exchange chromatographies. Different physical and chemical properties were then analysed: an SDS-PAGE electrophoresis revealed an average M_r of 28.8 kDa for procerain, putting it in the common molecular mass range of 20–35 kDa for plant cysteine proteases. No carbohydrate could have been determined by the phenol sulfuric acid method [45] thus differing procerain from glycoproteins. An isoelectric point of 9.32, defined by isoelectric focusing on polyacrylamide gel, indicates a predominance of basic amino acid residues. Total amino acid analysis revealed the presence of seven cysteines, one free and six forming disulfides [46], eight tryptophan and twenty tyrosine residues. In addition to proteolytic activity against different natural substrates such as casein, azocasein, azoalbumin and haemoglobin, procerain also showed hydrolytic activity against synthetic substrates. The enzyme's activity was best over a broad pH 7.0–9.0 range and together with its high stability with respect to temperature, denaturants and organic solvents, this makes it a very useful tool for food and pharmaceutical industry.

1.5. Cardenolides

Since the 1960s it has been known that the principal toxic components in *C. procera*, capable of causing death in mammals when reaching high concentrations, are cardenolides [47]. Chemically, cardenolides, cardiac glycosides named after their capacity to influence heart beat, are 5βH,14β-hydroxy α,β-unsaturated γ-lactones widely distributed in plants. In 1983, Erdman was among the first to measure individual nutrient and cardenolide concentrations in unextracted as well as in extracted *C. procera* [48]. Several cardenolides were positively identified: ascleposide, calactin, calotoxin, calotropin, calotropagenin, coroglaucigenin, proceroside, usharidin and uzarigenin. Recently, with the aim to use cardenolides as potential anti-cancer agents, the list of cardiotonic glycosides found in *C. procera* has been enlarged by 2-oxovoruscharin **02**, a derivative of voruscharin [49]. However, due to its high toxicity observed in mice, chemical modifications were necessary in order to improve tolerance *in vivo*. The outcome, named UNBS1450 **01**, not only showed reduced toxicity, but also a severely increased therapeutic anti-cancer activity [50].

Fig. 2 gives a short overview of how *C. procera* can be used in pharmaceutical science, either by applying rather simple methods, to extract for example latex, or by more sophisticated approaches seeking to isolate specific compounds like cardenolides.

2. Bioactivity of cardenolides

2.1. Effect of cardenolides on the sodium pump

Frequently named sodium pump, the Na,K-ATPase is an integral-membrane protein with both an enzymatic and a transporter function: in the conformational state E1, the phosphorylated protein binds three intracellular Na^+. These Na^+ ions are then released into the extracellular fluid, in exchange to 2 external K^+ ions and phosphate formation (state E2). With the Na^+/K^+ gradient being implicated in the regulation of animal cell volume, in lyses prevention, in membrane potential control, in pH homeostasis and in energy delivery for ion transport, the sodium pump is essential for cell viability and is thus ubiquitously expressed throughout the different tissues [51,52].

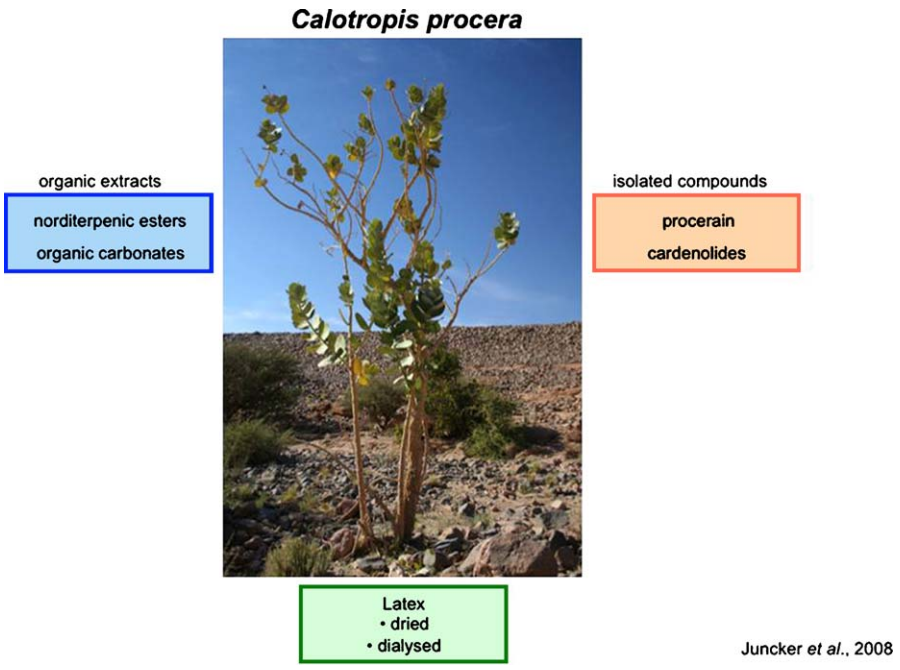

Fig. 2. *Calotropis procera* cartoon showing the three main approaches for using the plant's intrinsic pharmaceutical potential.

The Na,K-ATPase is a heterodimer formed of an α and β subunit. The α subunit, with a molecular mass of 106 kDa, contains a ten segment transmembrane domain (M) and three different cytosolic domains, namely a nucleotide binding domain (N) binding ATP, a phosphorylation domain (P) and an actuator domain (A). The abundantly glycosylated β subunit of 40 kDa has a single transmembrane segment, a short intracellular N-terminal and a larger extracellular site. In contrast to the catalyzing α subunit, the β subunit is required for the biogenesis and activity of the holoenzyme. A third subunit FXYD has been reported to be associated to the α and β subunits in some tissues.

Endogenous and exogenous cardiac glycosides, for example cardenolides, act by binding to extracellular H_1–H_2, H_3–H_4 and H_5–H_6 loops of the catalytic α subunits of sodium pumps. It had been shown that these cardenolide compounds are tightly fixed to the E2 conformational enzyme state, leading to inactivation [53]. E2-phosphoenzyme-cardiotonic compound complexes were found to be formed irreversibly. With varying K_d values, the inhibition complexes are probably internalized and degraded. Inhibitors of the Na^+,K^+ transporter lead to an increase of the cytosolic Na^+ concentration and are thus influencing the thermodynamics of the Na^+,Ca^{2+} exchange pump finally resulting in an increased Ca^{2+} concentration in sarcoplasmic reticulum [54] (Na^+-Lag hypothesis).

However, recent studies suggest that this Na^+ pump inhibition is not necessary to obtain an inotropic effect on myocytes [55]. By interacting with cardiac glycosides, the Na^+–K^+ pump complex has been shown to act as a signalosome. Various intracellular signaling pathways are responsive to cardiac glycoside binding; Ca^{2+} could play the role of a second messenger, the phosphatidylinositide 3′-kinase and protein kinase B can be activated as well as the SRC-EGFR-RAS-RAF-ERK cascade. In this way, cardiac glycosides may influence cell proliferation, cell differentiation and finally cell death by apoptosis (Fig. 3).

2.2. Cardenolide derivatives and their structure–activity relationship

Recently, Toxicaroside D **03**, isolated via bioassay-guided fractionation from a ethanolic stem extract of the rainforest tree *Antiaris toxicaria*, exhibited cell proliferation inhibition in different cancer cell lines together with 10 other cardenolides purified from the same source. Concerning structure–activity relationship, a reduction of 19-CHO and mono-glycosidation of the 3-hydroxy group resulted in a higher cytotoxicity to different cancer cell lines by factor 2 and over 100 respectively. In contrast, a β-hydroxylation of carbon 12 reduced the induction of apoptosis [56]. However, an additional acetoxy group did not modify the cytotoxicity [57,58]. These observations had been confirmed in a previous publication [57]. Structural modification of the core unit as an additional alcohol group or an epoxy group at C8 or a double bond between C16 and C17 led to a decrease of cytotoxicity [58]. Additionally, Ueda et al. isolated 15 cardenolides from the Vietnamese medicinal plant *Streptocaulon juventas*. They proved that di-glycosidation did not alter or even decrease the activity. Hence, the hydroxyl groups at C1 and C5 were important for the anti-proliferative property. These authors concluded that bioactivity of the isolated cardenolides-set depends on the hydrophilicity of the sugar unit linked to C3-OH because a free hydroxyl C2′ and acetylating of C3′OH reduced the cytotoxicity [57].

The research group of O'Doherty developed a novel stereoselective synthetic method to glycosylate the aglycone core units of cardenolides. The comparative study of the synthesized mono-, di- or tri-saccharide derivatives **05**–**07** of digitoxigenin **04** showed that the linkage of a monosaccharide increased the bioactivity [59], which confirmed the previously described studies by Yao and Kadota [56,57]. The higher anti-proliferative properties of the tri-saccharide derivatives of digoxin and gitoxin underlined this result [60]. In detail, the stereocenter at C2′ of the sugar unit plays a crucial role in cytotoxicity, as the unnatural epimers of the cardenolides loose almost their complete apoptotic activity against cancer cells [61].

The anti-inflammatory bioactivity of cardenolides proved to be sensible on chemical modifications of the core unit. Although, an additional acetoxy function at C16 does not alter the observed properties, an additional alcohol group at C8, modification of the C14 hydroxyl group or a double bond between C16 and C17 resulted in a lower anti-inflammatory activity [58].

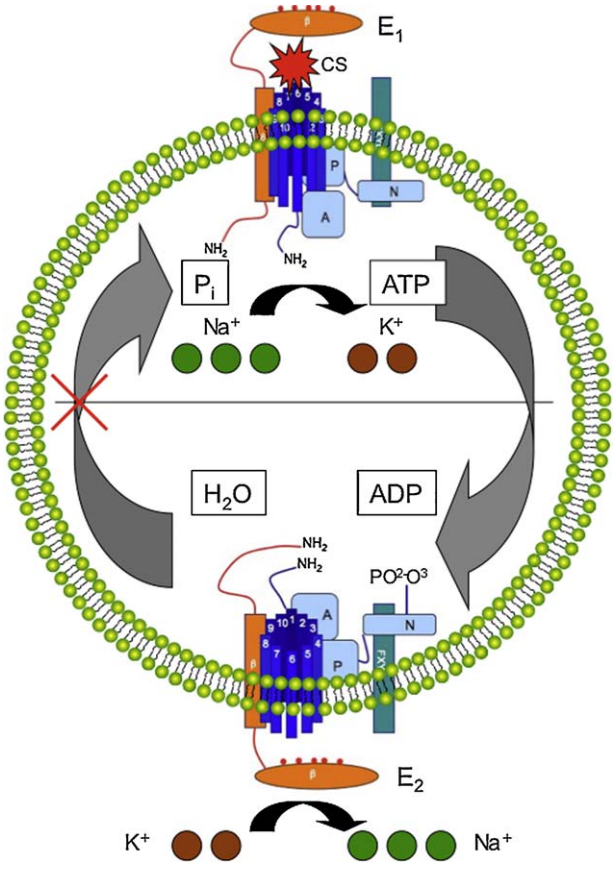

Fig. 3. Schematic representation of Na^+/K^+ ATPase inhibition by cardiotonic steroids. Two conformationally different states are known for the Na^+/K^+ ATPase: in E1, enzyme phosphorylation on the phosphorylation domain P enables intracellular binding of 3 Na^+ in exchange to 2 K^+, thus leading to state E2. The 3 Na^+ are released in the extracellular fluid whereas 2 new K^+ are imported. Through hydrolysis, the enzymatic complex returns to state E1 with P_i release. Cardiotonic steroids (CS) like cardenolides become tightly bound to the different extracellular loops (H_1–H_2, H_3–H_4, H_5–H_6) of the enzymatic α subunit. The holoenzyme is blocked in state E2, no further ion exchange is possible.

A low anti-proliferative activity is not necessary for the reversal of multi-drug resistance (MDR) as one cardenolide, even exhibiting a cytotoxicity at a concentration range up to 77 mM, might serve as potential future lead MDR-cancer reversal agent due to the calcein accumulation in MDR human ovarian cancer 2780AD cells [58]. This compound had been isolated first by Neumann and its structure determination had been carried out by the research teams of Reichstein and Yamauchi [62–64] (Figs. 4 and 5).

Interestingly, in the case of the hemi-synthetic derivative **01** of 2″-oxovoruscharin **02**, the reduction of the carboxyl function into a primary alcohol did not result in a higher cytotoxicity but the *in vivo* toxicity was reduced by factor 10 [49] (Fig. 4).

The design of novel anti-cancer drugs derived from natural products is still a matter in the development of potent cancer therapeutics. There is still a need and potential in further investigation of natural sodium pump inhibitors.

2.3. Pharmaceutical activity of cardenolides

With chronic heart failure being a widespread health problem in modern society, there is an urge for new therapy drugs. According to today's knowledge, arterial hypertension and coronary artery disease are among the main causes for the development of heart failure diseases. Constant exposure to high concentrations of hypertension-producing hormones like epinephrine, drives both, cardiac and arterial myocytes into molecular remodeling for example by internalizing β-adrenergic receptors, by altering G protein expressions, by lowering K^+ channel expressions and by increasing Na^+/Ca^{2+} pumps, thus favoring severely reduced intrasarcoplasmic Ca^{2+} concentrations [65].

Exogenous as wells as endogenous cardiac glycosides act according to the Na^+-Lag hypothesis: as seen before, partial inhibition of the Na^+–K^+ pump by these cardiac glycosides results in higher Na^+ concentrations leading to increased Ca^{2+} concentrations thus increasing cardiac output. Certain vertebrates endogenously produce cardiac glycosides with a steroid core, namely the cardenolides Ouabain and Digoxin as well as the Bufadienolides Marinobufagenin, Telocinobufagin and 19-Norbufalin. Moreover, since the 1990s there is increasing interest in the possible role of the sodium pumps in myocytic hypertrophy [66]. On *in vitro* myocyte cultures, inhibition of Na^+,K^+-ATPases by cardiotonic compounds like ouabain resulted in increased contractility, growth, protein synthesis, early proto-oncogene responses, AP-1 and NF-κB transcription factor activations thus explaining the beneficial effect of digitalis, another cardiotonic steroid, on heart failure. Despite being the oldest drug in cardiovascular medicine, digitalis is still being used in contemporary heart failure treatments.

Due to cardiotonic steroid-induced signaling pathways, the various endogenously synthesized cardiac glycosides constitute a new class of steroid hormones, regulating blood pressure, arterial tension and insulin release.

Because of the evidence that cardiac glycosides might influence cell proliferation and differentiation and that cancer cells have enhanced Na^+,K^+ activity, further studies trying to elucidate the effects of cardiac glycosides on tumor growth came up. In 2001, Stenkvist discovered a reduced death rate (6% versus 34%) of breast cancer patients when treated for a long period (22 years) with digitalis glycosides [67]. In the same context, leukemia has been shown to be less frequent in a group of patients treated with cardiac glycosides for heart failure. Also bufalin, a bufadienolide cardiac glycoside, has been used for ages in Chinese medicine to treat hepatocarcinoma and leukemia. Meanwhile, numerous studies indicated that cardiac glycosides are able to block several cancer cell types in the G_2/M cell cycle phase [68,69]. In order to further evaluate the cytotoxic effects of cardiac glycosides, it is of great importance to develop non-cardiotonic cardiac glycosides. UNBS1450 **01**, a semi-synthetic derivative of 2-oxovoruscharin **02**, one of the first non-cardiotonic compounds, first described in 2005, just entered phase I of clinical trials (Table 1).

3. UNBS1450 and its anti-cancer activity

3.1. The impact of UNBS1450 on the Na^+K^+-ATPase isozymes

Several isozyme forms are known for Na^+,K^+-ATPases, depending on tissue and cell type: 4α and 3β isoforms have been identified so far. Until today, severely increased expression of the α1 subunit has been associated to the clinical patterns of non-small cell lung cancers (NSCLC) and glioblastoma [70,71].

For both studies, sodium pump inhibition assays were performed using a heterologous protein expression system. Given the advantage of producing high amounts of recombinant Na^+/K^+-ATPases, baculovirus-transfected Sf-9 insect cells expressing α1β1, α2β1 and α3β1 isozymes were used to analyse the inhibitory capacities of several cardenolides, among which UNBS1450 **01**. When compared to ouabain and digoxin, UNBS1450 **01** showed a greater Na^+/K^+-ATPase isozyme inhibitory potential. The α3β1 dimer was mostly affected by an UNBS1450 inhibition,

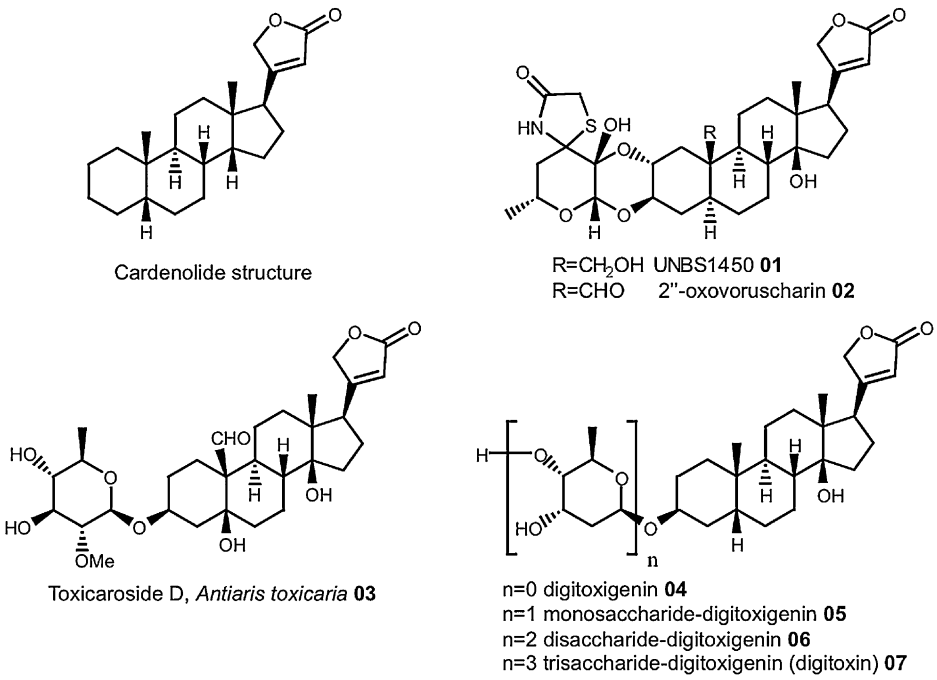

Fig. 4. Chemical structure of a small selection of natural sodium pump inhibitors and its analogues.

with a determined K_i of 3.2 nM. The $\alpha 2\beta 1$ and $\alpha 1\beta 1$ isozymes were also inhibited by UNBS1450 **01**, but to a lesser extent, with K_i values of 15 and 160 ± 70 nM respectively. Compared to classic cardenolides, the double-linked sugar moiety as well as the trans-conformational steroid structure seem to further potentiate the inhibitory activity of UNBS1450 **01**, rendering this cardenolide especially promising for additional anti-cancer studies based on modified cell proliferation, differentiation and cell death by inhibited sodium pump driven signaling.

According to recent findings, sodium pump signaling through UNBS1450 **01** binding leads to disorganization of the actin cytoskeleton, increased non-apoptotic cell death by autophagy, down-regulation of c-Myc in cancer cells, NF-κB deactivation and general modifications of the nucleolar morphology.

3.2. The impact on the NF-κB signaling pathway

As many typical cancer cell lines, A549, non-small cell lung cancer cells, show constitutive NF-κB pathway activation, and thus become able to escape cell death by apoptosis, necrosis, autophagy, senescence, mitotic catastrophe and paraptosis [72]. NF-κB activation favors multi-drug resistant cancer development.

Mijatovic et al. were among the very first to investigate a possible repressive potential of cardiotonic steroids and especially cardenolides on constitutive NF-κB activation [73]. In their work they used a new hemi-synthetic derivative of 2-oxovoruscharin **02**, a compound recently identified by themselves in *C. procera* and isolated by liquid chromatography from methanolic extracts from the root barks of *C. procera*. In contrast to stereotype cardenolides, the chemically modified 2-oxovoruscharin named UNBS1450 **01** shows a double-linked sugar moiety and a trans-conformational steroid core.

As a first result, they managed to show that UNBS1450 **01** is able to influence NF-κB activity in human A549 cells. In fact, a 24-h treatment with UNBS1450 **01** with a concentration of 10 nM was sufficient to point out induced accumulation of I-κBβ, however not of I-κBα. For the latest, phosphorylation levels were decreased after 4–8 h post-treatment. The expression of cdc34, an enzyme implicated in I-κBα ubiquitination, was also decreased. Further NF-κB binding (Trans-AM) as well as NF-κB reporter gene (luciferase reporter plasmid construct pNF-κB-Luc) assays confirmed the *in vitro* inhibitory potential of UNBS1450 **01** on A549 tumor cells. Secondly, preliminary *in vivo* studies revealed increased survival of A549 orthotopic xenograft-bearing nude

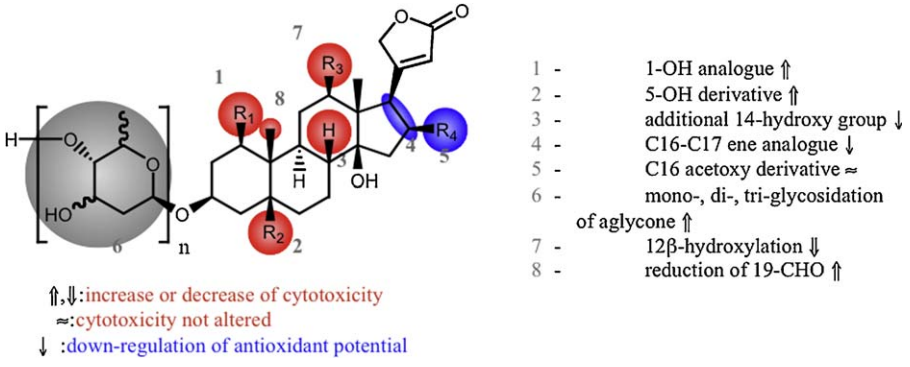

Fig. 5. SAR studies on analogues of natural sodium pump inhibitors. Summary of structure–activity relationship (SAR) results of natural sodium pump inhibitors and derivatives concerning the observed anti-cancer cytotoxicity and anti-oxidant properties. The pharmacophores identified during the studies are highlighted in colour [49,56–64].

Table 1
Main endogenous and exogenous cardenolides and their pharmaceutical activity.

Digoxin Marinobufagenin 19-Norbufalin Ouabain Telocinobufagin	Partial inhibition of the Na^+–K^+ pump leading to increased intracellular Ca^{2+} concentrations; increased contractility and metabolism in myocytes *in vitro*	Endogenous
Bufalin	Beneficial effect in hepatocarcinoma and leukemia treatment	Exogenous
Digitalis	Still in use for heart failure treatments	
UNBS1450	One of the first non-cardiotonic cardenolides; promising anti-cancer potentials	

mice when treated with different concentrations of the maximum tolerated dose of UNBS1450 **01**.

3.3. The effect of UNBS1450 on cell proliferation and cancer

Starting with *in vitro* anti-proliferation assays in 2005, Van Quaquebeke et al. analysed cytotoxicity levels of several cardenolides, among which UNBS1450 **01**, on 57 human cancer cell lines; for each compound average IC_{50} values were determined by MTT assays after 72 h of incubation. As reference drugs, a tubulin inhibitor (taxol) and a topoisomerase I inhibitor (SN-38) were chosen. Concerning the individual sensitivity of each human cancer cell line, significant differences were observed between UNBS1450 **01**, taxol and SN-38 suggesting a different antitumor mechanism for UNBS1450 **01**. In terms of average growth inhibition however, UNBS1450 **01** was proven to be as potent as taxol, with a mean IC_{50} of 2.7 nM for UNBS1450 **01** and 2.5 nM for taxol. SN-38 was significantly less potent. Furthermore, when applicated on multi-drug resistant tumor bladder cells, UNBS1450 **01** showed rather astonishing capacities in inhibiting cell growth: vincristine and adriamycin resistant tumor cells forced taxol to reach concentrations of 10,000 nM to become cytotoxic whereas UNBS1450 **01** became effective at 45 nM. Complementary to these data, UNBS1450 **01** also showed cytotoxic effects on rat glioma, mouse mammary carcinoma and melanoma cells but at a far less extent when compared to UNBS1450 **01** anti-tumor effects in human cancer cells. This feature could relate to the double mutation observed in the α1 subunit of rodent sodium pump.

More recently, the observed findings were confirmed: UNBS1450 **01** is also capable of repressing *in vitro* growth of A549 tumor cells as well as growth and migration of glioblastoma cell lines.

UNBS1450's *in vivo* anti-cancer activity has been investigated so far by tumor growth analysis in xenograft models. Generally, by administering UNBS1450 **01** chronically to nude mice bearing orthotopic xenografts of NSCLC and glioblastoma cell lines, cytotoxicity of this hemi-synthetic cardenolide has been determined by intraperitoneal or per os injections. In both cases, the maximum tolerated dose (MTD) had been previously elucidated acutely in healthy mice. When compared to classical cardenolides such as ouabain and digoxin as well as to its precursor, 2-oxovoruscharin **02**, UNBS1450 **01** showed significantly increased MTD values: for ouabain and digoxin, MTD values were 5 and 10 mg/kg respectively, whereas for UNBS1450 **01** the MTD value raised up to 120 mg/kg. As a result, chronic administration of UNBS1450 **01** was realized with different concentrations, from a 32- to an 8-fold reduced MTD [73]. Interestingly, UNBS1450 **01** at concentrations from 5 to 20 mg/kg was proven to be able to increase grafted mice survival. In control mice, about 20 days were sufficient to bring the mouse population down to 45%. In contrast, mice which had been chronically administrated (12 times) with 10 mg/kg of UNBS1450 **01** showed best results, with a population down to 45% only after 40 days. Higher concentrations (20 mg/kg) seemed to be less encouraging since the outcome was less promising than for 10 mg/kg, probably due to slight toxic effects.

Comparable results were obtained for *per os* administrations of UNBS1450 **01**, nevertheless requiring higher dosages. Oral uptake of UNBS1450 **01** at 80 mg/kg significantly increased mouse survival. Whereas the 50% survival barrier was broken shortly after 20 days post-tumor graft in control mice, with 80 mg/kg of UNBS1450 **01** this point was delayed to day 40.

A principal characteristic of many cancer cells is their high invasiveness; glioblastoma cells for instance are active, "self-propelled" brain cancer cells, highly capable of invading brain tissue by shape and volume modifications [74]. Today it is believed that ion transport plays an important role in enabling cancer cell migration [75]. Until very recently, the impact of the Na^+,K^+-ATPase in cancer cell migration remained unexplored. In early 2008, Lefranc et al. tried to establish a link between glioblastoma cell migratory invasion, sodium pump and cardenolide administration [71]. At first, they demonstrated that in human glioblastoma cells the α1 subunit of the Na^+,K^+-ATPase is overexpressed when compared to normal human brain tissue. Using immunofluorescent staining, they were able to show that α1 subunits co-localize with caveolin-1 in the cytoplasmic protrusions of U373-MG glioblastoma cells.

Additionally, UNBS1450 **01** was able to exert a cytotoxic effect on human U373-MG cells; the same was true for rat C6 cells, but to a lesser extent. For U373-MG, concentrations of around 100 nM of both UNBS1450 **01** as well as ouabain rendered cell survival nearly impossible. For rat C9 cells, ouabain was practically ineffective whereas high concentrations reaching 10 μM were required to completely abolish cell viability. An impact of UNBS1450 **01** on intracellular ATP concentrations in human glioblastoma cells had then been suggested. Next, computer-assisted phase-contrast microscopy analysis permitted to gain further insights in cellular proliferation, morphology and motility as well as on ion homeostasis. They discovered that in the presence of 10 nM UNBS1450 **01**, U373 cells are well capable of undergoing mitosis, but fail to divide into daughter cells and to form lamellipodia. Nevertheless, the observed, round-shaped U373 cells remained alive for several days (mitochondria staining). However, contrary to a possible hypothesis, the administration of 10 nM of UNBS1450 **01** did not result in either an increased Ca^{2+} nor an increased Na^+ concentration, which at the same time constitutes one of the major advantages of UNBS1450 when compared to other cardiac glycosides like digitalis. Increasing intracellular Ca^{2+} favors arrhythmias in otherwise healthy people. The observed changes were probably due to irreversibly modified actin cytoskeleton, thus explaining reduced migratory capacities of these human glioblastoma cells (Fig. 6).

3.4. UNBS1450-induced cell death

3.4.1. By apoptosis

It has been reported in literature that cardiac steroid glycosides are *a priori* potentially able to induce cell death by apoptosis. For example it has been observed in vitro that different leukemia cell lines such as THP-1 [76], K562 [77], HL-60, U937 and ML1 [78] undergo apoptosis when treated with different concentrations of Bufalin or Digoxin. However, when treated with rather low nanomolar doses of these cardiac glycosides, it might preferentially stimulate differentiation and proliferation. It is believed that nanomolar concentrations lead to low-frequency oscillations of Ca^{2+} concentrations favoring NF-κB activation and protection

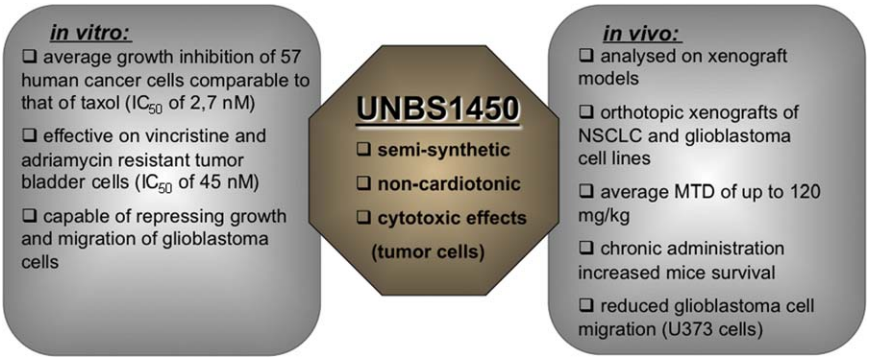

Fig. 6. *In vitro* and *in vivo* effects of UNBS1450.

against apoptosis whereas higher concentrations would lead to apoptosis due to sustained, high levels of Na^+ and Ca^{2+}[79].

In contrast to many other cardiac steroids, UNBS1450 **01** does not seem to induce cell death by apoptosis *in vitro*, at least when working with adherent cell lines (evidence provided by flow cytometry analyses after propidium iodide and Annexin V staining). To our knowledge, the entire studies on UNBS1450's anti-proliferative and anti-cancer activity has been carried out on solid tumor cell lines. UNBS1450 **01** stimulation has been shown to induce non-apoptotic cell death [71,80].

3.4.2. By autophagy

Recent studies on UNBS1450-induced cell death claim pro-autophagic effects to be responsible for the observed cytotoxic potential [71]. It has been reported that during autophagy, besides apoptosis, one of the two self-destructive processes, intracellular organelles are enclosed in characteristic double- or multi-membraned vacuoles which finally fusion with lysosomes thus leading to degradation [81]. Together with apoptosis, cellular "self-eating" has the aim to eliminate superfluous, damaged or aged cells or organelles like mitochondria and endoplasmic reticulum. Moreover, being implicated in macromolecule catabolism, autophagy helps cells in overcoming stress situations, thus avoiding probable cell death by apoptosis for instance. Depending on the situation, autophagy sometimes constitutes an alternative pathway to apoptosis, sometimes a possibility to escape apoptosis.

In 2006, first studies indicating UNBS1450-induced autophagy in cancer cells appeared [80]. Working on NSCLC cell lines, cell death was indeed relevant after treatments with UNBS1450 **01**: a 24 h treatment with 100 nM of UNBS1450 reduced cell viability to about 70%, when prolonged to 72 h, 10 nM were sufficient to obtain similar results. Consequently, PARP cleavage analysis permitted to exclude cell death through apoptosis, as PARP remained intact. Complementary performed Tunel assays ultimately confirmed the absence of apoptotic cell death. Cell cycle analysis revealed increased percentages of cells in S and G_2 phases whereas microscopy images clearly showed severe cytoplasmic vacuole formation. Further Mijatovic et al. made clear that UNBS1450 **01** induces Hsp70 downregulation thus rendering possible lysosomal membrane permeabilization, typically inhibited in lung cancer cells by heightened levels of Hsp70 which constitute a prosurvival function. Lysosomal permeabilization is a known indicator of cell death through autophagy; these findings of 2006 then strengthened the hypothesis of non-apoptotic cell death by "self-eating" in UNBS1450 treated cancer cells.

In the same context UNBS1450 **01** has been assigned to induced cell death through autophagy in different human glioblastoma cell lines [71]. In a first step, acridine orange

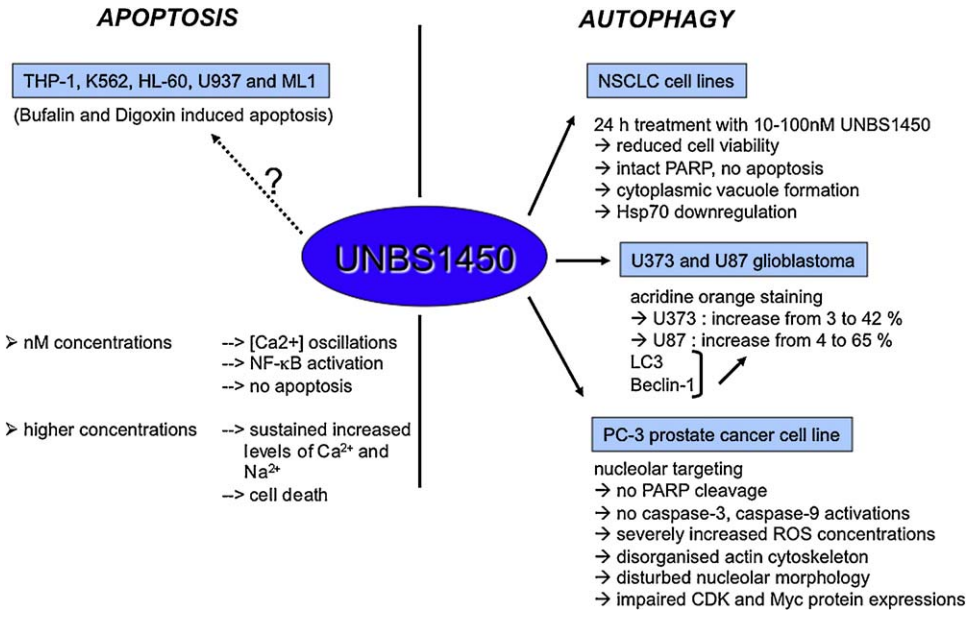

Fig. 7. Apoptosis and autophagy-related cell death induced by UNBS1450.

staining was used in order to quantify acidic vesicular organelles whose abundance is tightly associated to autophagy. Indeed, the presence of nanomolar UNBS1450 **01** significantly increased staining in U373-MG and U87-MG cells (from 3 to 42 and from 4 to 65%). At the same time, the expressions of LC3 and beclin-1, two specific autophagy markers, were found to be stimulated by UNBS1450 **01**.

Besides lung cancer and glioblastoma cell lines, UNBS1450 **01** has also been found to induce probably autophagy-related cell death in human prostate cancer cell lines by severe nucleolar targeting [82]. In fact, UNBS1450 **01** efficiently inhibited apoptosis-resistant PC-3 prostate cancer cell growth at 100 nM, without caspase-3 and caspase-9 activation and PARP cleavage. Intracellular Ca^{2+} concentrations were not modified, in contrast to cellular ROS load. Not only did UNBS1450 **01** (10 and 100 nM) severely increase ROS production, computer-assisted phase-contrast microscopy revealed that it again completely disorganized the actin cytoskeleton as seen before and disturbs nucleolar morphology. The nucleolus domain constitutes the site of ribosome biogenesis and is thus indispensable to cell survival. It has been reported that nucleolar regions are of great importance in cancer cells as it has been proven that tumor cells retain tumor suppressors, like p53 and MYC for instance, inside the nucleoli. The observed impairments of CDK and Myc protein expressions might thus be mechanisms of nucleolar disorganization and induced autophagy (Fig. 7).

4. Conclusion

Taken together, these results suggest that the novel hemisynthetic cardenolide UNBS1450 **01** from *C. procera* is able to repress cancer cell proliferation and even induce cell death. Despite the fact that the plant's beneficial effects on many different pathologies have been admitted many centuries ago, the real breakthrough is still to come. Both, the entire latex as well as organic extracts, might well have proven their usefulness in treating different clinical entities, only recent advances in research revealed the true potential lying within *C. procera*. By chemically modifying a novel cardenolide isolated from this tropical plant, researchers discovered with UNBS1450 **01** a new and very promising weapon in cancer therapy.

In contrast to many other anti-cancer agents, UNBS1450 **01** does not induce apoptosis but autophagy, at least in solid tumor cells. Essentially by deactivating NF-κB, down-regulating Hsp70 and by modifying nucleolar structure, UNBS1450 **01** leads to vacuole formation, actin cytoskeleton impairment and finally cell death.

Further insights, particularly concerning its usefulness in leukemia treatment, still remain to be investigated.

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