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RESEARCH

Two calix[4]pyrroles as potential therapeutics for castration-resistant prostate cancer

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Summary
Macrocyclic compounds meso-(p-acetamidophenyl)-calix[4]pyrrole and meso-(m-acetamidophenyl)-calix[4]pyrrole have previously been reported to exhibit cytotoxic properties towards lung cancer cells. Here, we report pre-clinical in vitro and in vivo studies showing that these calixpyrrole derivatives can inhibit cell growth in both PC3 and DU145 prostatic cancer cell lines. We explored the impact of these compounds on programmed cell death, as well as their ability to inhibit cellular invasion. In this study we have demonstrated the safety of these macrocyclic compounds by cytotoxicity tests on ex-vivo human peripheral blood mononuclear cells (PBMCs), and by in vivo subcutaneous administration. Preliminary in vivo tests demonstrated no hepato-, no nephro- and no genotoxicity in Balb/c mice compared to controls treated with cisplatin. These findings suggest these calixpyrroles might be novel therapeutic tools for the treatment of prostate cancer and of particular interest for the treatment of androgen-independent castration-resistant prostate cancer.

Keywords calix[4]pyrroles · Prostate cancer · Chemoresistance · Tumour drug resistance · New anticancer drugs · Drug development · Cancer

Introduction

Prostate cancer (PCa) is considered one of the 'big killers', accounting for 1.2 million cases and 359,000 deaths in 2018 [1]. According to GLOBOCAN 2018, PCa is the second most frequent cancer in men and the fifth cause of death worldwide. Its prevalence is higher in developed countries and its mortality rate is strongly correlated to age [1]. PCa affects mainly patients with significant comorbidities. These epidemiological features provide an estimate of the socioeconomic impact of this disease. Androgen deprivation therapy (ADT) represent the cornerstone of PCa treatment as the initial response rate is about 80%. During ADT, disease progresses and develops distant metastases in 35% of patients treated for local PCa, while 10–20% of the patients with advanced stage of the disease become refractory to ADT [2] evolving castration-resistant prostate cancer (CRPC). Notably, the development of CRPC is mostly associated with poor prognosis and overall survival time of about 14 months [3]. Although, by 2019 several clinical trials regarding the therapeutic management of PCa were concluded with positive results, to date the advanced and aggressive variants of CRPC remain incurable [4].

Calix[4]pyrroles (C4PYs) are macrocycles (heterocyclophanes) containing four pyrrole units linked by quaternary carbon atoms [5]. Unlike porphyrins, they do not have a planar shape and an extended delocalized electronic system. The pyrrole NH units provide an array of hydrogen-bond donors that can effectively interact with a variety of hydrogen-bond acceptors (anions as well as neutral molecules), thus forming supramolecular complexes in which the selectivity (towards different acceptors and their binding strengths) can

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be extensively modulated by decorating the calixpyrrole core-structure with ancillary chemical moieties [6–13].

Although C4PYs are known since more than a century [Baeyer, A. (1886), Ueber ein Condensations product von Pyrrol mit Aceton. Ber. Dtsch. Chem. Ges., 19: 2184–2185. https://doi.org/10.1002/cber.188601902121], their use in medicinal chemistry has been pioneered by our group through a rather serendipitous approach. Looking for a novel nanovector to properly deliver cytotoxic drugs directly to cancer cells, we initially reported the ability of *meso-p*-aminophenylcalix[4]pyrrole to form a cytotoxic *trans*-Pt(II) complex (**215** in Fig. 1) in which the calix unit appears to assist the delivery of the toxic metal to DNA via the preliminary binding of the phosphate residues [14]. Later on, it has been demonstrated how some C4PYs derivatives exhibit cytotoxic actions against selected cell lines by acting as ionophores capable of altering chloride transport across cell membranes [15]. In parallel, we reported that *meso*-octamethyl-calix[4]pyrrole (**100** in Fig. 1) acts as an antagonist of the G-Protein Coupled Estrogen Receptor 30 (GPER/GPR30) in different model systems, such as breast tumour cells and cancer-associated fibroblasts [16] hence exhibiting a biological action that is associated with its ability to interact with specific proteins rather than acting just as ionophores. To our minds, these results indicated that C4PYs deserve additional investigation as potential drugs in their own right, rather than as merely vectors of biologically active species. In this context we showed that the C4PY derivatives **107** and **563** (Fig. 1) exhibit very different cytotoxicity towards different cell lines [17, 18]. All together, these observations prompted us to further investigate **107** and **563** searching for cancer types for which they could be useful drug candidates. Hereby, we report cytotoxic effects of **107** and **563** on two androgen-independent PCa cell lines (DU145 and PC3).

Materials and methods

Chemistry, cell lines and culture

Calixpyrrole derivatives **107** and **563** were prepared as previously reported [17] and their purity checked.

Prostate cancer (PCa: PC3, DU145 and LNCaP), small cell lung cancer (SCLC: DMS-79, IST-SL1, IST-SL2), ovarian serous adenocarcinoma (A2774) and glioblastoma (U251) cell lines were purchased from the ICLC (Biological Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy), and cultured respectively in RPMI (Pan Biotech, Aidenbach, Germany) or DMEM (Sigma-Aldrich, Milan, Italy), both supplemented with 10% fetal calf serum (Euroclone, Milan, Italy), 2 mM L-glutamine (Euroclone, Milan, Italy), and 1% penicillin–streptomycin (Euroclone, Milan, Italy) at 37 °C in a 5% CO_2 incubator.

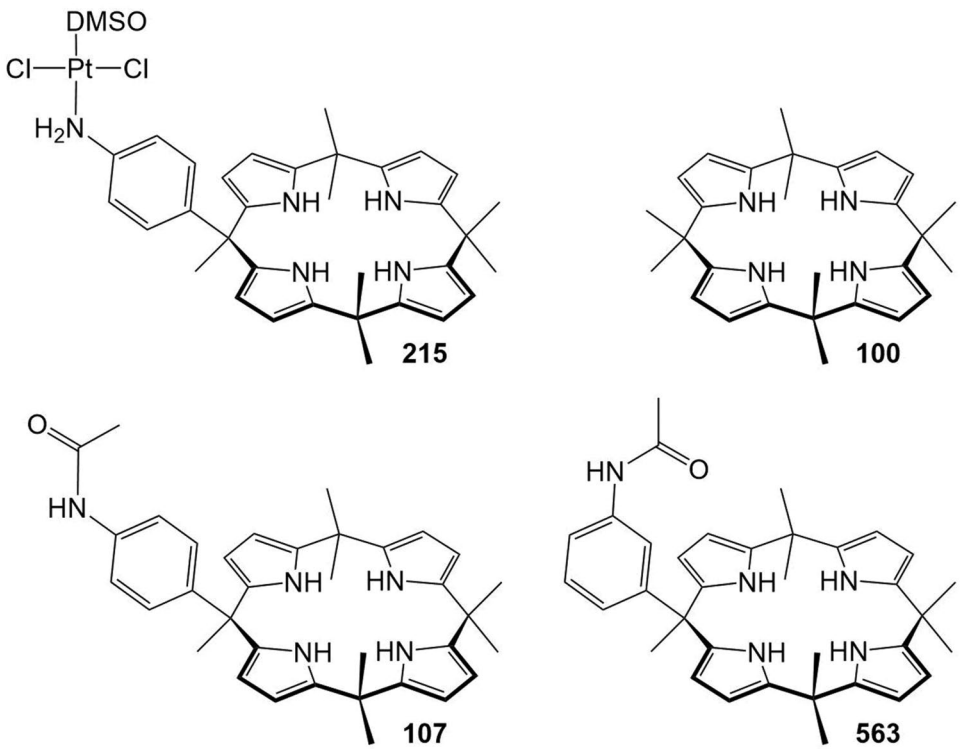

Fig. 1 Structural formulae of calix[4]pyrroles **215**, **100**, **107** and **563**

MTT assay

We evaluated cytotoxicity by MTT assay; cancer cell lines were seeded in 96-well plates and treated with various concentrations (up to 100 μM) of **107** or **563** for 48 h. Tests for each compound was conducted independently at least three times. The half maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism® software (GraphPad Prism® 6.01, GraphPad Software, Inc., CA, USA).

Annexin-V analysis of apoptotic cells

Apoptosis analysis was performed using Muse™ Annexin V & Dead Cell Assay. PC3 and DU145 cells were seeded at a density of 2×10^5 per well in 1 mL of culture medium DMEM (Sigma-Aldrich, Milan, Italy), and treated with **107** or **563** at IC_{50} concentrations for 48 h. Samples were stained at room temperature in the dark for 20 min using Muse™ Annexin V & Dead Cell Reagent (Millipore Merck, Vimodrone Milan, Italy).

Multicaspase assay

PC3 and DU145 cells (1×10^6 cells/well/2 mL culture media) were seeded at 37 °C in a 6-well plate and allowed to reach 70–80% confluence, then treated with **107** or **563** for 24 h at IC_{50} concentrations as previously described [19]. Muse™ multicaspase assay kit (Millipore Merck, Vimodrone Milan, Italy) was used for the detection of multiple caspase activation (caspase-1, -3, -4, -5, -6, -7, -8 and -9), following manufacturer's instructions. The percentage of cells with multicaspase activity was subsequently examined using Muse™ cell analyzer (Millipore Merck, Vimodrone Milan, Italy) flow cytometer.

Cell invasion assays

Cell invasion assays were carried out in Matrigel chambers (BD Bio Coat) as previously described [20]. PC3 and DU145 cells were seeded (3×10^4 cell/well) in the Matrigel chamber in serum-free medium with 0.1% DMSO as control or with 1 μM solutions of **107**, **563**, or etoposide. Invasion assay was carried out using medium supplemented with 20% FBS as a chemo attractant for 48 h. PC3 and DU145 cells that invaded the lower chamber were fixed with 100% methanol and stained with 1% toluidine blue in 1% borax. The invasion index was determined by cell count as the ratio of cells treated with **107** or **563** compared to untreated cells that invaded the Matrigel chamber.

Ex-vivo toxicity of 107 and 563

The toxicity of **107** and **563** was examined against Peripheral Blood Mononuclear cells (PBMCs) from healthy volunteers (22–55 years). PBMCs were cultured in medium supplemented with interleukin-2 (IL-2) and seeded in a 96-well microplate (4×10^5 – 5×10^4 cells/200μL/well). The two compounds were added to the cell cultures at final concentrations ranging from 1–300 μM. For this purpose, **107** and **563** were added in identical volumes of DMSO solutions of appropriate concentrations in order to have a final concentration of DMSO 0.1% in the cell culture medium in all cases. The cells were incubated at 37 °C for 48 h in a humidified 5% CO_2 incubator. Cell cytotoxicity was evaluated by the trypan blue dye exclusion technique.

Experimental design for the study on animals

After an acclimation period, Balb/c mice were randomly divided into 3 groups of 6 mice each. The first group received a subcutaneous injection with 50 mg/kg b.w. of **107** dissolved in an olive oil/DMSO mixture. The positive control group was treated with intraperitoneal injections of cisplatin (CDDP) (15 mg/kg b.w.) dissolved in a DMSO/PBS solution, the negative control group was administered with 5% DMSO in PBS. In all cases the amount of DMSO administered to the mice was below 0.01% of the body weight. Animals were killed 72 h after the injection. Body weight was measured before and after treatment with **107** and CDDP. The major organs (spleen, liver, lung, brain, and kidney) were harvested, inspected and weighed on an electronic balance after blotting the blood and body fluids on filter paper. The organ coefficient was calculated as the ratio of organ weight (in mg) per 10 g of animal weight. Blood samples were collected from the scarified animals and immediately placed in heparin anticoagulant tubes. Samples were then centrifuged at 1,500 rpm for 7 min. Plasma was obtained and stored at -20 °C for biochemical parameters analysis.

Genotoxic effect of 107

Alkaline comet assay was carried out as reported previously [21]. Spleens and kidneys of Balb/c mice treated with **107** or CDDP were individually homogenized with 10 mM Tris–HCl (pH 7.4) and centrifuged at 12,000 rpm at 4 °C for 25 min. The cellular supernatants were used to examine DNA damage. Cellular suspensions were embedded in agarose gel, lysed, and subjected to electrophoresis. The total score of DNA damage was calculated as reported [22].

Table 1 Estimated Concentration (d, μM) of C4PY derivatives **107** or **563** needed to induce a 50% decrease in cellular viability. NT = not tested. *IC_{50} reported in our previous study (Geretto et. al. [17])

	PC3	DU145	DMS-79	IST-SL1	IST-SL2	A2774	U251
107	4.9	9.0	d > 100	d > 100	d > 100	10 < d < 20	50 < d < 100
563	10.8	9.9	d > 100	d > 100	d > 100	10 < d < 20	NT
	H727*	**A549***	**MCF-7***	**MDA-MB-231***	**SKOV3***	**U87MG***	-
107	d < 5	d < 5	20 < d < 30	10 < d < 20	20 < d < 30	50 < d < 100	-
563	d < 5	d < 5	10 < d < 20	10 < d < 20	5 < d < 10	50 < d < 100	-

Histopathology

For histological analysis, kidney sections were prepared as previously described [23]. Organs were collected from the 3 groups and: a) fixed with 10% phosphate-buffered neutral formalin; b) dehydrated in graded alcohol (75–95%), and c) embedded in paraffin. Slices (5 μm thick) were stained with hematoxylin and eosin (H & E) and analyzed by optical microscopy (Leica DMI 4000 B; Leica Microsystems, Wetzlar, Germany).

Determination of liver and kidney function indexes

Aspartate transaminase (AST), alanine transaminase (ALT), and creatinine (CR) levels in plasma were evaluated by clinical chemistry analyzer COBAS C × 72. 4.15.

Results

Cytotoxicity of C4PYs 107 and 563 in different human cancer cell lines

The viability of PCa (PC3 and DU145), SCLC (DMS-79, IST-SL1, IST-SL2), ovarian serous adenocarcinoma (A2774) and glioblastoma (U251) cell lines were evaluated by MTT assay after 48 h treatment with C4PYs 107 or 563. The highest cytotoxic activity was observed against PCa cell lines (Table 1). Dose–response curves for the cytotoxic effects of 107 and 563 against PC3 and DU145 are shown in Fig. 2. The IC_{50} for 107 and 563 were 4.9 μM and 10.8 μM for PC3 cell line and 9.0 μM and 9.9 μM for DU145, respectively. A lower anti proliferative potential was recorded for ovarian and glioblastoma cell lines. No significant cytotoxic effect was detected for SCLC cell lines.

Apoptosis induction in PC3 and DU145 cell lines

We analysed the apoptotic potential of **107** and **563** in PC3 and DU145 cell lines at the IC_{50} concentrations determined using MTT. The Annexin V/7-AAD assay confirmed the IC_{50} obtained through the MTT assays and the prevalent apoptotic mechanism of cell death in PC3 and DU145 (Supplementary Figs. S1 and S2 respectively).

We also used the Muse™ multicaspase (Millipore Merck, Vimodrone Milan, Italy) assay kit to gain an insight into the apoptosis mechanism of action. Caspase activation (caspase-1, 3, 4, 5, 6, 7, 8, and 9) was evaluated. After 24 h **107** (IC_{50}) and **563** (IC_{50}) were able to increase caspase activation in PC3 (19.47% and 20.52% respectively) and DU145 (32.15% and 10.99% respectively) (Supplementary Figs. S3 and S4). Thus, for DU145 cells, **107** induced a higher caspase activation than **563**. All indicated values are referred to the DMSO negative control.

Invasion inhibition of PC3 and DU145 cell lines treated with 107 and 563

We evaluated the inhibitory effects of **107** and **563** on the invasive ability of PC3 and DU145 cells by Matrigel invasion assay. Based on previous results, this test was conducted using the C4PYs derivatives at 1 μM concentration. Compared to control cells treated with DMSO alone (0.1%), **107** and **563** displayed a remarkable inhibitory effect against PC3

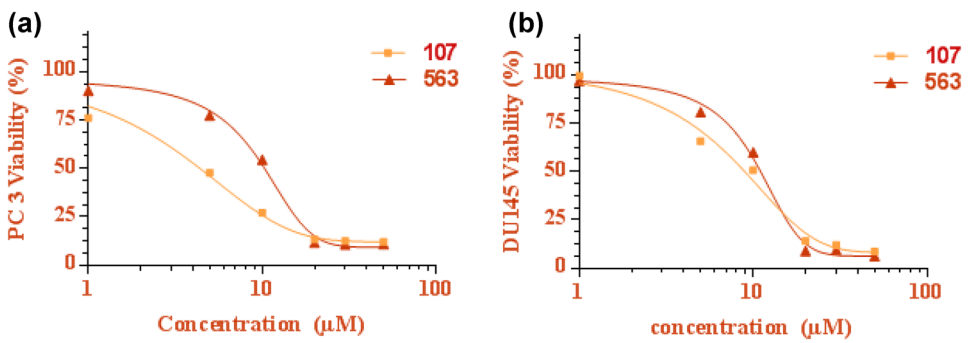

Fig. 2 Cytotoxicity dose–response curves of **107** and **563** for PC3 (**a**) and DU145 (**b**) cell lines using MTT assay. Data represent the mean ± standard deviation of the mean of 3 independent experiments

and DU145 cell invasiveness. For PC3 33.6% and 43.9% respectively (Supplementary Fig. S5), and for DU145 14.6% and 80.7% respectively (Supplementary Fig. S6), in comparison to the negative control (100%). Notably, the inhibitory activity of **107** and **563** was found to be more effective than 1 µM etoposide, an antineoplastic agent used as the positive control [24]. This in vitro invasion assay suggests that C4PY **107** and **563** inhibit the metastatic potential of PCa cells.

Ex-vivo toxicity of 107 and 563

The toxicity of **107** and **563** were determined against PBMCs collected from healthy volunteers. The PBMCs were treated with **107** and **563** at 1–300 µM concentrations. After 48 h, the viability of both controls and treated cells was determined by trypan blue dye exclusion. The results showed no cytotoxic effect on lymphocytes compared to the negative control at any of the tested concentrations (Fig. 3).

Effect of C4PY 107 on mice weight organ coefficient and morphologies

The toxicity study was limited to **107** as this compound was found to be the most cytotoxic in cancer cells compared to **563**. After 72 h from the cutaneous administration of **107** to Balb/c mice no significant changes in body weight, mental state, food, and water consumption could be detected compared to the negative control. In contrast, mice treated with CDDP appeared to suffer from exhaustion (behaviour), and showed a significant body weight loss (Supplementary Fig. S7). After CDDP administration, mice showed normal average weight for liver, lung, brain, and heart while we observed a significant reduction of spleen and kidney coefficients (Supplementary Table ST2). In contrast, the administration of **107** did not affect the organ coefficients nor spleen and kidney morphology (Fig. 4).

Effect of 107 on ALT, AST and creatinine serum levels

Transaminases and creatinine serum levels were measured in the three groups of mice treated with **107**, CDDP, and DMSO controls after 72 h. While significantly increased functional biomarkers were observed in CDDP-treated mice, there was no statistically significant difference between the negative control and mice treated with **107**, indicating no hepato- or nephro-toxicity induced by the tested compound (Supplementary Fig. S8). The effects of CDDP on these biochemical parameters, are consistent with those reported previously [25].

Genotoxic effect of C4PY 107: Comet assay

DNA damage induction was evaluated using the alkaline comet assay (Fig. 5). The total score of DNA damage showed no significant differences between spleen and kidney cells of mice treated with **107** and negative controls. However, we detected a significant increase in total DNA damage of spleen and kidney in mice treated only with CDDP.

Kidney histopathology

Histopathological analysis of the mice kidneys revealed normal glomerulus and tubules with regular morphology in both negative controls and **107**-treated animals. By contrast, CDDP induced damage in the kidneys characterized by renal tubule necrosis (Fig. 6).

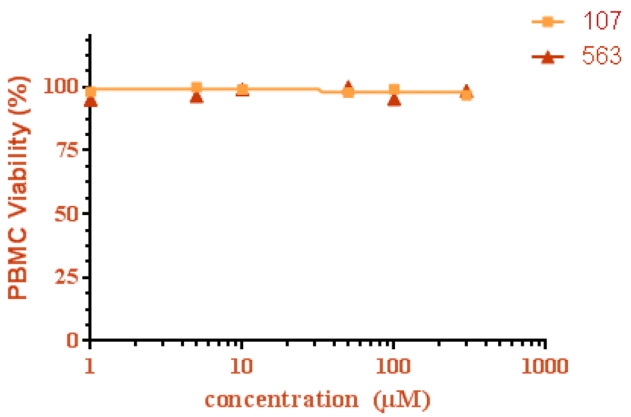

Fig. 3 Cytotoxic effect of **107** and **563** on human PBMCs. Cell viabilities were calculated for **107** and **563** at 1.5, 10, 50, 100, and 300 µM concentrations at 48 h and are normalized to 0.1% DMSO (control)

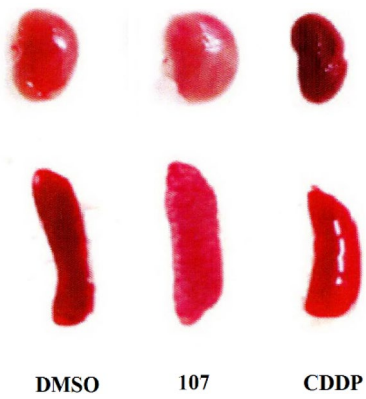

Fig. 4 Effect of **107** and CDDP on kidney and spleen morphology: DMSO negative control with normal organ morphologies; **107**-treated; CDDP-treated. All random samples from each group

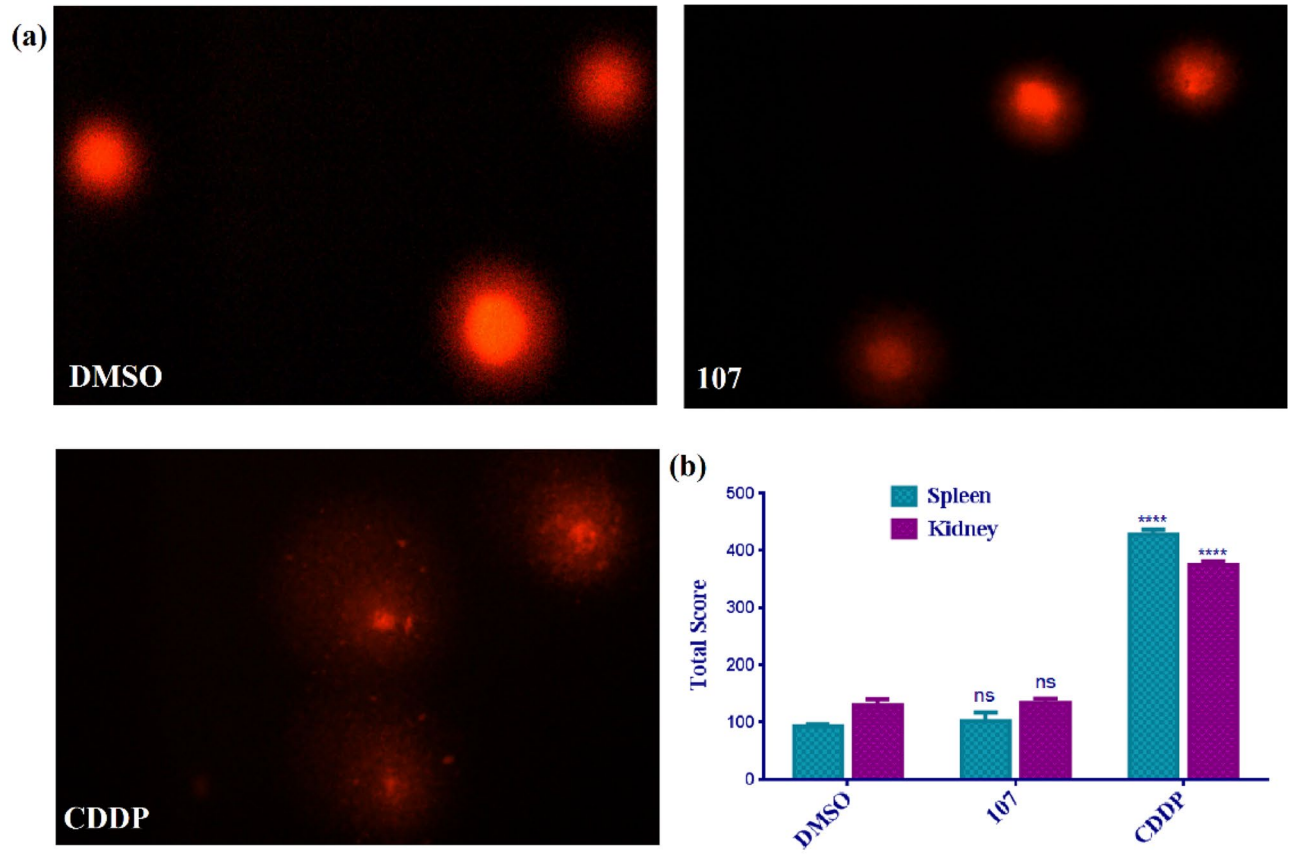

Fig. 5 **a** Photographs for the comet assay evaluation of DNA damage in mice kidney cells: NC negative control (DMSO 0.1%), CDDP-treated, **107**-treated. **b** Histogram reports ex-vivo comet score of the spleen and kidney cells. ****$P < 0.0001$ compared to the negative control group that received equal dosage of DMSO; CDDP was used as positive control

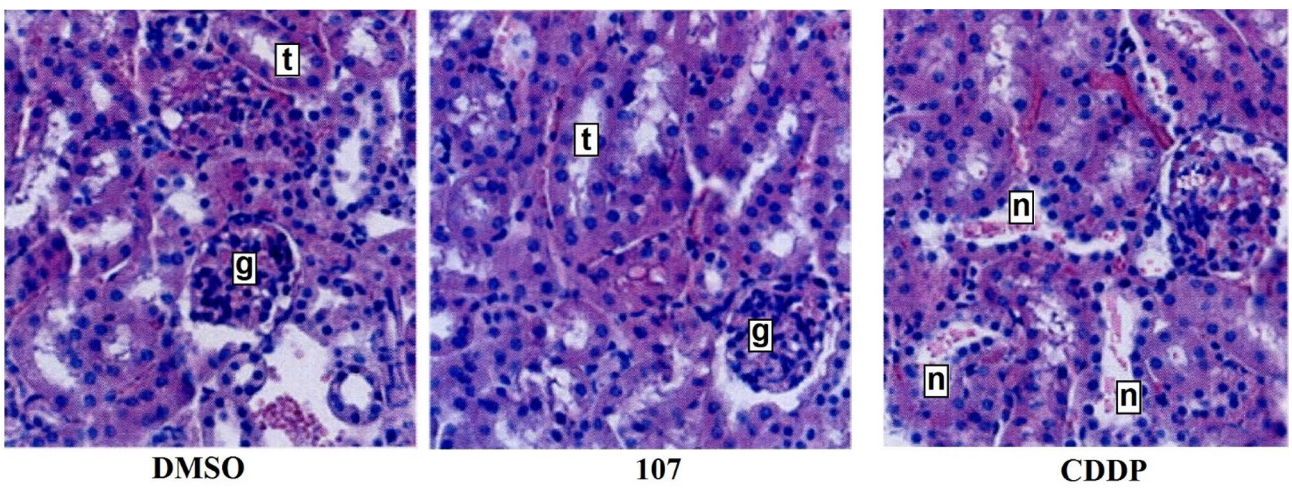

Fig. 6 Histology of kidney samples: DMSO negative control; **107**-treated mouse; and CDDP-treated mouse. Normal histological structure of the tubules (t) and glomeruli (g) are observed in the negative control and in **107**-treated animals; necrosis in tubules (n) is evident in CDDP-treated mice (c); hematoxylin–eosin staining, magnifications: × 40

Discussion

Crucial steps in the development of a new drug include the determination of its toxicity and of the molecular mechanism of action (i.e. the biological target). For cancer drugs, which should impair or kill cancer cells, toxicity is a requirement that should be evaluated in relative terms with respect to non-targeted cells, hence selectivity is paramount. Indeed, our study indicates considerable selectivity of **107** and **563** towards different cancer cells lines: powerful cytotoxic action against non-small cell lung cancer A549 and PCa (PC3 and DU145), modest activity against ovarian cancer A2774 and SKOV3, and negligible activity against glioma U251, and SCLC (IST-SL1, IST-SL2, and DMS-79) cell lines. This selectivity of action leads us to the hypothesis that the formation of DNA adducts we have previously reported [17] might not be the primary mechanism of action. Data on the human apoptotic proteins array (checking expressions of 43 proteins) was followed by a qRT-PCR to investigate the apoptosis pathway (see Supplementary Information and Supplementary Fig. S9). We demonstrated that **107** and **563** modulate the apoptotic protein and gene expressions including Bcl-2 and caspase 3 in A549. These data triggered our interest in apoptosis including the abnormal expression of Bcl-2 that increases anti-apoptotic potency and chemoresistance.

We have also evaluated LNCaP cells, another PCa cell line that, unlike DU145 and PC3, express the AR receptor, and that was considered an in vitro PCa model for the phase prior to the establishment of anti-androgen resistance. Only modest activities (IC_{50} of approximately 30 μM) were observed for both compounds, probably due to the inherently lower proliferation rate of LNCaP cells with respect to DU145 and PC3 cells (data not shown). Indeed, some drugs which act on DNA or its repair mechanisms usually work better on faster replicating cells than on slower growing cells such as LNCaP. There is a great deal of documentation to support this, including our previous paper on **107** and **563** against A549 [17].

We considered various drugs commonly used in PCa treatment to be used as the 'in house' positive reference. Taking into account the above cited modulation of Bcl-2 and caspase 3 in A549 cells, we referred to a paper [26] in which the authors investigated the effects of Bcl-2 expression status on the susceptibility of DU145 cells to docetaxel (a taxane) and CDDP. Bcl-2 expression levels were unrelated to the susceptibility of DU145 cells to docetaxel. The sensitivity of DU145 cells to CDDP was reported. In a xenograft mouse model, over-expression of Bcl-2 drastically decreased the sensitivity of DU145 cells to CDDP. However, there was no change in the response to docetaxel. Interestingly, in this study docetaxel barely induced caspase-3/7 activation in controls or in Bcl-2-overexpressing DU145 cells. These findings indicate that the antitumor effects of docetaxel on DU145 cells are independent of both Bcl-2 and pro-apoptotic caspases. Therefore, although taxanes are first-line chemotherapeutic agents for PCa, in light of our previous study and that of Ogura, we were interested in using CDDP as a positive blank in our toxicity tests. Moreover, CDDP is a convenient reference compound because it is a conventional chemo-drug endowed with significant hepato- and nephro-toxicity [27].

We tested in vitro and ex-vivo toxicity of **107** and **563** on human PBMCs and on mice. There was no cytotoxic effect on lymphocytes compared to the negative control, at any of the concentrations tested. In vivo studies evidenced that 72 h after the subcutaneous administration of **107** at 50 mg/Kg dose there were no significant changes in Balb/c body weight, behavior, food and water consumption compared to the negative controls. In contrast, mice treated with 15 mg/kg of CDDP suffered from fatigue as well as exhibiting a significant loss of body weight. Transaminases (ALT and AST) and creatinine levels were not significantly different from negative controls, showing that **107** is neither hepato- nor nephro-toxic. Moreover, the comet assay showed no genotoxicity and histopathology of the kidney also exhibited normal glomerulus and tubule morphology.

The cell lines that derive from bone metastasis (PC3) and brain metastasis (DU145) are extensively used in research as a powerful tool for antitumoral drug discovery and for understanding the molecular pathways implicated in PCa anti-androgen resistance [28]. Both C4PY derivatives induced apoptosis after 48 h, as demonstrated by the Annexin V tests in both PCa cell lines.

The growth and survival of PCa cancers depend on androgens, male steroid hormones. The principal androgens are testosterone and dihydrotestosterone (DHT) which activate and bind to the androgen receptor (AR) [29]. High expression of AR is observed in most patients with castration resistant prostate cancer (CRPC). Moreover, the expression of AR-regulated genes that is restored in patients that received ADT implies that AR transcriptional activity is reactivated [29]. In particular, there is evidence that after ADT, a high concentration of DHT, the most potent AR agonist, can be produced by androgen precursors of adrenal origin [30, 31]. Consequently, molecular targets used to treat CRPC are not only AR antagonists but also androgen synthesis inhibitors. Other strategies in CRPC include targeting the mechanisms of survival (PI3K/AKT/mTOR), DNA repair, immune checkpoints, and stress-response.

In a previous study we demonstrated that C4PY **100** is a novel antagonist ligand of the Membrane Estrogen Receptor GPER [16] also providing an example for the binding of a calixpyrrole with a protein. The interaction of calixpyrrole

derivatives with amino acids is also well documented [32]. Thus the observed selectivity, together with the numerous and considerable changes in miRNA expression determined in A549 cells point to a mechanism of action that is more specific than the exclusive formation of DNA adducts or a general impairment of cell homeostasis due to the transport of chloride ions across membranes, as previously suggested for other calix[4]pyrrole derivatives [15]. Recently, polymers capable of binding polyanionic proteins were reported to kill PCa cancer cells [33], and indeed **107** and **563** are anion binders.

The ability of **107** and **563** to inhibit PC3 and DU145 cell migration, at concentrations that are significantly lower than their IC_{50}, is also an unprecedented discovery of this study with groundbreaking implications for the potential exploitation of the biological activity of calixpyrroles. The significance of this result lies in the invasiveness of PCa for which metastasis development is associated with poor prognosis and overall survival [34].

Conclusions

This work demonstrates that **107** and **563** are potentially useful new drugs or lead-compounds for the development of novel tools against CRPC. Despite the limitation that the details of their mechanism of action have still to be uncovered, a pragmatic observation of data presented here cannot but underscore this statement. All experiment conducted so far in order to uncover any significant toxicity (in vitro or in vivo) towards heathy cells or tissues, has given no indication of any adverse effects at the dosages that were effective against cell lines PC3, and DU145 in this study, and H727 and A549 in the previously reported one [17]. We are undertaking experiments by differential mass spectrometry on treated vs. non-treated cells to further characterize the genes and signaling pathways that are altered by **107** and **563** in different in vitro cancer models. The identification of specific alterations should allow us to define the cellular phenotype sensitive to these C4PY derivatives and pave the way to additional therapeutic options not only for CRPC patients but also for other neoplastic diseases.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10637-022-01294-8.

Author contributions C.R.., Conceptualization**;** I.B.T, M.P., P.B., F.H.K.., S.F., and B.B. Investigation; I.B.T, L.C.G., P.B., B.B. and A.I. Formal analysis; I.B.T, G.C.V. and C.R Writing—original draft; P.B., E.I., G.C.V. and C.R. Writing—review & editing; A.I. and C.R. Methodology and validation; C.R., Funding acquisition and Supervision.

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Data availability statement Any additional information will be made available by the authors, without undue reservation, to any qualified researcher upon request.

Declarations

Ethics statement All experiments on Balb/c mice (22–26 g) were performed following the European Communities Council Directive (86/609/EEC; November 24, 1986) regulating the welfare of experimental animals, and experiments were approved by the Life Sciences and Health Research Ethics Committee (cer-svs) of the Institute of Biotechnology (University of Monastir, Tunisia; ethical approval no. 2019/02/I/CER-SVS/ISBM; 9 January, 2019). Mice were housed under standard conditions in an accredited pathogen-free facility. All animals were provided access to filtered water and standard rodent chow ad libitum.

Conflict of interest C.R. is member of the Advisory Board of GIAM PHARMA International S.A.R.L., Monthey (CH). Other authors declare any conflict of interest.

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