Research article

Differential mechanism of action of the CK1ε inhibitor GSD0054

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Abstract

In the current study, we explored for the first time, the mechanism of action of the new Casein kinase 1 ε (CK1ε) selective inhibitor GSD0054. Although GSD0054 behaved as a selective CK1ε inhibitor in enzymatic assays, we studied whether this inhibitory activity also occurred inside the cells. The effects of GSD0054 on β-catenin expression and disruption of cell cycle progression were studied in the human breast cancer cell lines MDA-MB-453 (β-catenin negative) and T-47D (β-catenin positive). We also performed molecular modeling studies using computational docking against CK1ε to explain and predict the mechanism of action of this compound. Moreover, the commercially available CK1ε inhibitor PF-4800567 and the CK1δ/ε inhibitors PF-670462 and IC261 were also studied for comparison purposes. GSD0054 showed anti-proliferative activity against MDA-MB-453 and T-47D cells despite the fact that MDA-MB-453 cells do not possess active β-catenin. However, selective cell killing occurred in the more resistant, β-catenin active, T-47D cells. CK1ε was confirmed as a cellular target, although other targets or alternative mechanisms of action could possibly explain the anti-proliferative activity in MDA-MB-453 cells.

Keywords

Synthetic lethality; Casein kinase inhibitors; Wnt/beta-catenin; Anti-beta-amino alcohols; Breast cancer

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1. Introduction

Casein kinase 1 ε (CK1ε) belongs to a highly conserved and ubiquitously expressed family of serine/threonine protein kinases. At least, seven CK1 isoforms (α, β, γ 1-3, δ and ε) have been identified in vertebrates [1]. With the exception of CK1β which has been found solely in cows, all the other CK1 isoforms are expressed in humans. Besides other physiological implications, CK1 isoforms are important in cancer progression in diverse types of tumors [2]. In particular, changes in the activity or expression levels of CK1ε can contribute to the development of cancer [3]. Moreover, other CK1 isoforms did not induce growth arrest, demonstrating the role of CK1ε in promoting tumor cell proliferation [4].

CK1ε is the protein encoded by the *CSNK1E* gene and behaves as an essential modulator of the Wnt/β-catenin signaling pathway, which is implicated in embryonic development, stem cell biology and diseases like cancer. This pathway is regulated at different levels by a broad range of effectors including CK1 members CK1α, δ and ε [5] and its deregulation has been implicated in a variety of human cancers. CK1ε is required for the survival of breast cancer subtypes that rely on aberrant β-catenin activity. Active myristoylated CK1ε is able to induce malignant transformation by stabilizing β-catenin and activating the transcription of Wnt target genes [6], which include several proto-oncogenes like colon cancer related *c-myc, n-myc*, ovarian adenocarcinoma related *FGF9* and breast cancer-related *LBH*. The list of target genes gives an idea of the relevance of this pathway and the potential of its regulation. Therefore, targeting key mediators of the Wnt signaling pathway such as CK1ε may inhibit tumor progression [7].

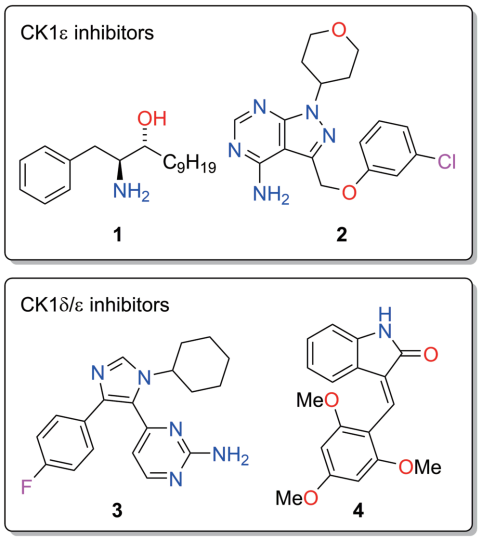

Fig. 1. Chemical structures of ICK1ε GSD0054 (1) and PF-4800567 (2); and ICK1δ/ε PF-670462 (3) and IC 261 (4).

As part of our interest in the discovery of new anti-proliferative agents, we have reported a new CK1ε selective inhibitor (ICK1ε) [8]. Thus, a small and structure-biased library of enantiopure *anti*-β-amino alcohols was prepared in a straightforward manner by a sim-

plified version of the Reetz protocol [9]. For most active compounds, the antiproliferative activity against a panel of human solid tumor cell lines yielded GI_{50} values in the range 1-20 μM. We designed a computational approach to predict the molecular target(s) based on the chemical structure and the antiproliferative activity. This method pointed to kinases as plausible candidates. The experimental determination of the interaction with 456 kinases indicated that these *anti*-β-amino alcohols behave as selective ICK1ε. The *anti*-β-amino alcohol GSD0054 (1) (Fig. 1) was selected as the lead drug.

In the current work, we describe the first findings on the mechanism of action of GSD0054 (1). Although we have unveiled its role as a growth inhibitor in cell cultures and CK1ε selective inhibition in kinetic assays, we attempted to find out if these activities correlate. Herein, we report the preliminary studies on the mechanism of action of GSD0054 (1) and compare the results with those of the ICK1ε PF-4800567 (2); and the CK1δ/ε dual inhibitors (ICK1δ/ε) PF-670492 (3) and IC 261 (4) (Fig. 1).

2. Materials and Methods

2.1. Chemicals

GSD0054 (1) was prepared as described earlier [8]. PF-4800567 (2), PF-670462 (3) and IC 261 (4) were purchased from Sigma-Aldrich.

2.2. Cell lines and culture

The breast cancer cell line MDA-MB-453 was provided by CEAMED S. A. (Tenerife, Canary Islands). The breast cancer cell line T-47D, the non-small human lung cancer cell line SW1573 and its P-gp overexpressing variant (SW1573/P-gp) were kindly provided by Prof. G. J. Peters (Cancer Center Amsterdam, Vrije Universiteit, Amsterdam, The Netherlands). Cells were grown in RPMI-1640 medium containing 5% heat- inactivated FCS, 2 mM L-glutamine, 100 U/mL of penicillin G and 0.1 mg/mL of streptomycin in a 37°C, 95% humidified atmosphere of 5% CO_2. Cells were maintained in culture in 100 mm cell culture dishes in growth medium (10 mL) and passaged twice weekly.

2.3. Antiproliferative assays

Cell line suspensions were counted with Moxi Z and diluted to reach the appropriate cell densities (SW1573 2,500 cells/well; T-47D, MDA-MB-453 and SW1573/P-gp 5,000 cells/well) for inoculation onto 96-well plates. After 24 hours, pure compounds were added. Each agent was initially dissolved in DMSO at 400 times the desired final maximum test concentration (100 μM). Control cell samples were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Compounds 1-4 were tested at serial dilutions (0.01-100 μM). Paclitaxel was used as a positive control and was initially dissolved in DMSO at a stock solution of 4 mM. Verapamil was used as a P-gp transport inhibitor and the stock solution was prepared in DMSO at 40 mM. Cell culture medium containing verapamil was prepared by adding the final concentration of 10 μM verapamil.

Drug incubation times were 48 hours, after which cells were precipitated with 25 μL ice-cold trichloroacetic acid solution (50% w/v) and fixed for 60 min at 4°C. Then, plates were rinsed with water, following which 25 μL of a sulforhodamine B (SRB) solution (0.4% w/v in 1% acetic acid) was added for 15 min. Unbound SRB was rinsed with 1% acetic acid. Bound SRB was dissolved with 150 μL of a 10 mM Tris solution (pH 10.5). The optical density of each well was determined at 530 and 620 nm using PowerWave XS microplate reader. Dual wavelength was used to reduce optical interference caused by scratches, fingerprints or other matter that absorb light equally at both wavelengths. The anti-proliferative activity, expressed as GI_{50} (50% growth inhibition), was calculated according to NCI formulas [10].

2.4. β-Catenin expression

Western blot analysis was used to determine the expression of β-catenin in T-47D cells. MDA-MB-453 cells were used as a negative control as these cells barely express detectable levels of nuclear (transcriptionally active) β-catenin [6]. Cell line suspensions of MDA-MB-453 and T-47D were counted with Moxi Z and diluted to reach the appropriate cell densities for the inoculation of 50,000 cells/well onto 6-well plates. After 24 hours, compounds 1-4 were added only to wells containing T-47D cells. Drug incubation times were 24 hours, after which time the growth medium was aspirated and cells were washed with PBS and treated with 1 mL of lysis buffer (25 mM Tris pH 8.0; 125 mM NaCl; 1% SDS; and 6.3 M urea). Lysates were collected in 1.5 mL microtubes and frozen until use. Cells floating in the growth medium were harvested in a different manner to prevent their loss; growth medium and PBS were collected together and then centrifuged at 1500 rpm for 2 minutes. The supernatant was removed before adding the lysis buffer to the cell pellet.

The amount of protein in cell lysates was quantified to ensure equal loading (20 μg of protein) onto the polyacrylamide gels. Samples were then mixed with 5 μL of loading buffer (4% SDS, 0.05% bromophenol blue, 20% glycerol and 2% β-mercaptoethanol in 100 mM Tris-HCl, pH 6.8), heated at 95°C for 5 min and loaded onto 8% acrylamide gels. Electrophoresis was performed at a constant voltage of 160 V (120 V first 15 minutes) for one hour. A molecular weight marker was also loaded in order to facilitate identification of molecular weights. Electroblotting of the proteins from the gel to the membrane took place at 160 V-300 mA for one hour. The membrane was then incubated with agitation in 4 mL milk over a period of 45 minutes to block membrane pores and then 1 μL of primary (mouse) antibodies (anti-β-catenin and anti-β-actin) were added and left over night. Secondary antibodies conjugated to horseradish peroxidase were used to analyze protein bands and quantified using ImageQuant.

2.5. Cell cycle

Changes in cell cycle were studied using the DNA dye propidium iodide (PI) and flow cytometry. Cells were seeded onto six well plates at a density of 50,000 cells/well. On the next day, compounds 1-4 were added to the respective wells and incubated for 24 hours. After treatment, cells were trypsinized, harvested, transferred to tubes and centrifuged at 300 for 10 min. Cell pellets were resuspended in 200 μL of cold PBS and fixed in 1 mL ice-cold 70% ethanol overnight at −20°C. Fixed cells were centrifuged and resuspended in 500 μL of PBS containing 0.1 mg/mL of DNAse-free RNAse. The mixture was incubated in the dark at 37°C for 30 min. After incubation, 5 μL of PI (0.5 mg/mL) were added at room temperature. Typically, 4500 events were collected using excitation/emission wavelengths of 488/670 nm for PI. Flow cytometric analysis of DNA content

was undertaken using Accuri C6. Cell aggregates were gated out and viable cells were selected based on size, complexity and DNA content such that the fractions of the cells in G_0/G_1, S, and G_2/M phase were calculated using cell cycle analysis software.

2.6. Reactive oxygen species (ROS) production

The level of ROS was determined using the ROS-Glo^{TM} H_2O_2 Assay (Promega Corporation, USA). Cells were exposed to compounds at the indicated doses for 48 h, after which time the non-lytic assay was performed following the manufacturer's instructions. Luminescence was measured on a Synergy HTX multi-mode microplate reader (BioTek, USA).

2.7. Docking

CK1ε structure (PDB ID: 4HNI) was downloaded from Protein Data Bank. We used HyperChem software to draw and optimize the molecular structures and OpenBabel application to turn the files into adequate format before exporting them to docking software Glide. We also performed docking calculations of compound IC261 against the crystal structure of β-tubulin (PDB ID: 3E22). For the purpose of the docking calculations, we removed ligands and non-essential water molecules present in the crystal structure and added polar hydrogen atoms to the protein chain before running any docking calculations.

3. Results

3.1. Antiproliferative effects in MDA-MB-453 and t-47D cells

In order to determine the antiproliferative activity (expressed as GI_{50}) of the inhibitors 1-4, we exposed MDA-MB-453 and T-47D cells to these compounds for 48 h. All inhibitors suppressed cell proliferation in both tumor cell lines (Table 1). Compounds 1-3 exhibited GI_{50} values in the range 2.4-79 μM, whilst inhibitor 4 was much more active with GI_{50} values in the range 32-87 nM. While inhibitor 4 constitutes the most potent compound of the series in all the tested tumor cell lines, compound 2 appears to be the less active in this context. Compound 1 displayed more potent inhibition than the selective inhibitor 2. The selective ICK1ε 1-2 showed stronger antiproliferative effect on MDA-MB-453 cells. In contrast, the ICK1δ/ε 3-4 were more active against T-47D cells. The ratios between GI_{50} values obtained for each compound are shown in Table 1.

Table 1. Anti-proliferative activity (GI_{50}) of ICK1ε drugs in MDA-MB-453 (β-catenin negative) and T-47D (β-catenin positive) cell lines[a].

	MDA-MB-453	T-47D	R[b]
GSD0054 (1)	2.4 ± 0.5	17 ± 1.7	7.1
PF-4800567 (2)	58 ± 11	79 ± 6.4	1.4
PF-670462 (3)	42 ± 6.5	11 ± 2.0	3.8
IC 261 (4)	0.087 ± 0.040	0.032 ± 0.007	2.7

[a]Values are given in μM and represent mean values of at least three independent experiments ± standard deviation.
[b]R is defined as the quotient of high and low GI_{50} for each compound against both cell lines.

3.2. Anti-proliferative effects against SW1573 cells

To determine whether or not P-gp could affect the activity of GSD0054, we determined the GI_{50} values after 48 h of exposure to ICK1ε 1-4 in wild type and P-gp- overexpressing SW1573 cells, and in the presence of the P-gp transport inhibitor verapamil (at a fixed concentration of 10 μM). The standard microtubule-interacting drug paclitaxel was used for comparison purposes. Table 2 shows the GI_{50} values obtained after 48 h of drug exposure. For better comparison of the data, we defined resistance factor (Rf) for a given compound as the ratio of GI_{50} values of the P-gp overexpressing and the wild-type cell line. In the absence of verapamil, we observed that ICK1ε 1-4 were not affected by P-gp overexpression (Rf = 0.6-3.2). These results indicate that the activity of ICK1ε 1-4 is not affected by the overexpression of P-gp. In addition, the dose response curves (Fig. 2) show that only GSD0054 (1) is able to induce net cell killing, which occurs at doses higher than 30 μM.

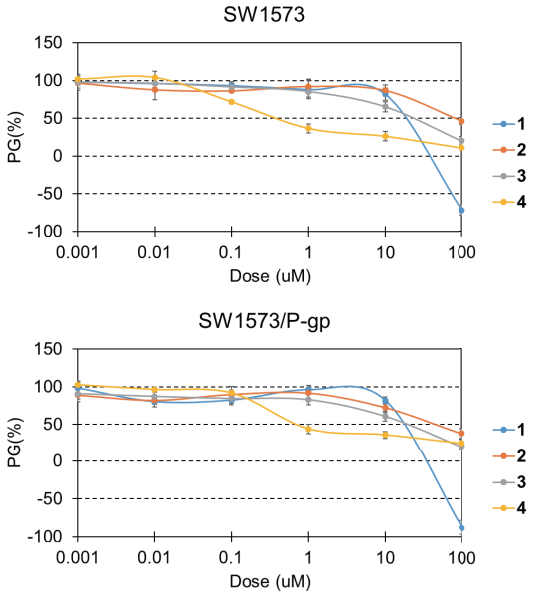

Fig. 2. Dose-response curves of SW1573 and SW1573/P-gp cells treated with ICK1ε 1-4 for 48 h.

3.3. Effects on β-catenin expression

As an indirect way to determine CK1ε inhibition, we evaluated the capacity of compounds 1-4 to reduce β-catenin expression in T-47D cells. MDA-MB-453 cells were used as a negative control since they barely express detectable nuclear β-catenin levels. Thus, T-47D cells were treated with compounds 1-4 at three drug concentrations, which were selected from the corresponding GI_{50} values (Table 1). The results are shown in Fig. 2. After 24 h of incubation, the drug GSD0054 (1) was able to reduce significantly the expression of β-catenin at concentrations of 20 μM ($\approx GI_{50}$) or higher. Compounds PF-4800567 (2) and PF-670462 (3) did not exert any inhibitory effect on β-catenin levels. However, treatment of T-47D cells with IC 261 (4) induced a reduction in the expression of β-catenin after 24 h of exposure at 100 nM ($\approx 3\times GI_{50}$).

Table 2. Anti-proliferative activity (GI_{50}) of ICK1ε in SW1573 and SW1573/P-gp cell lines[a].

	− Verapamil			+ Verapamil		
	SW1573	SW1573/P-gp	Rf[b]	SW1573	SW1573/P-gp	Rf
GSD0054 (1)	16 ± 2	16 ± 3	1.0	20 ± 1	17 ± 4	0.9
PF-4800567 (2)	76 ± 26	48 ± 24	0.6	85 ± 14	51 ± 25	0.6
PF-670462 (3)	19 ± 3	19 ± 8	1.0	21 ± 7	19 ± 6	0.9
IC 261 (4)	0.25 ± 0.03	0.79 ± 0.10	3.2	0.35 ± 0.03	0.86 ± 0.42	2.5
Paclitaxel	0.0029 ± 0.0009	4 ± 0.5	1379	0.0030 ± 0.0015	0.0036 ± 0.0015	1.2

[a]Values are given in μM and represent mean values of at least three independent experiments ± standard deviation.
[b]Rf represents the GI_{50} ratio between SW1573/P-gp and SW1573.

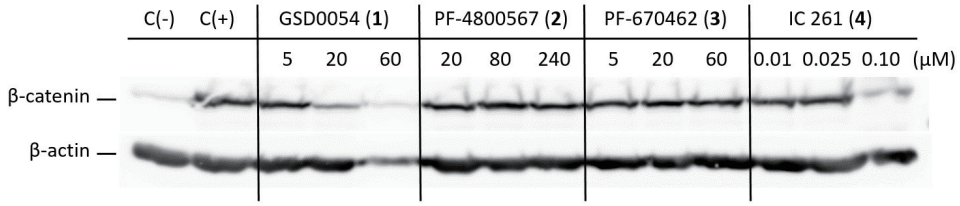

Fig. 3. Expression of β-catenin in drug-treated T-47D cells. Cells were exposed to compounds 1-4 for 24 h at the indicated doses. C (-): MDA-MB-453, negative control; C (+): T-47D, positive control. β-Actin signals allowed us to normalize the experiment.

Table 3. Docking scores of drugs 1-4 against CK1ε.

Compound	Score (kcal/mol)
GSD0054 (1)	−5.8
PF-4800567 (2)	−10.8
PF-670462 (3)	−6.5
IC 261 (4)	−5.9

3.4. Cell cycle analysis

We used flow cytometry to study the effect of inhibitors 1-4 on the cell cycle. Cells were exposed for 24 h to two drug doses (low and high), chosen based on the GI_{50} values (Table 1). The effect on the cell cycle was dose-dependent and varied for each compound (Fig. 3). Thus, GSD0054 (1) induced a notable accumulation of MDA-MB-453 cells at the G_1 phase and a slight increase in the sub-G_1 population was noted that reached 6.3% at the high dose. The effect of GSD0054 (1) on T-47D cells was different from that on β-catenin-lacking cells. There was a notable increase in the sub-G_1 population that peaked at about 41% at the high drug dose. In contrast, PF-4800567 (2) showed a quite similar effect on both tumor cell lines, leading to a marked accumulation of cells in the G_1 phase, concomitant with a decrease in the S and G_2/M phases. Moving on to PF-670462 (3), we observed a rise in MDA-MB-453 population at the G_1 phase when exposed to the compound, while the effect on T-47D cells remained unclear due to a possible aneuploid population. Importantly, compound IC 261 (4) exerted a very different effect on the cell cycle leading to an increase in the S and G_2/M populations in both cell lines.

3.5. ROS production

In order to gain an in-depth information about redox processes in the antiproliferative activity of ICK1ε 1-4, the ROS-Glo™ H_2O_2 assay was used. Importantly, the ROS-Glo™ assay does not use horseradish peroxidase (HRP), which is known to cause a high number of false hits, providing a simple format for both cell-based and enzymatic assays. Since various ROS are converted to H_2O_2 in the cell, and H_2O_2 is the longest-lived ROS, an increase in H_2O_2 can reflect a general increase in the ROS level. In particular, this experiment was applied to breast cancer cell lines MDA-MB-453 and T-47D, in the presence of model compounds at a low and a high dose (based on their GI_{50} values), and an incubation time of 48 h. The results shown in Fig. 5 evidence a dose-dependent increase of ROS levels in the β-catenin positive tumor cell line (T-47D) for all compounds except for ICK1ε 2. However, in MDA-MB-453 cells, no relevant increase in ROS production was observed.

3.6. Docking studies

We performed docking calculations against the binding site of CK1ε. The results for compounds 1-4 expressed as docking score (representing the variation in free energy of binding) are given in Table 3. Glide software provided energy scores of each compound; hence this value was a modality to predict how favorable the interaction between the protein and the ligand is. Thus, lower docking scores (more negative energy) indicate better interaction. As seen in Table 3, ICK1ε 2 displays the most favorable interaction with CK1ε, with a docking score of −10.8 kcal/mol. However, the remaining inhibitors showed docking scores in the range −5.8 to −6.5 kcal/mol. We also docked compound IC 261 (4) against β-tubulin either at the colchicine or the GTP site. The docking results indicate that IC 261 (4) has more favorable interactions with β-tubulin at the colchicine binding site (−6.2 kcal/mol) than at the GTP site (−4.5 kcal/mol). These results are in agreement with the reported experimental findings [7].

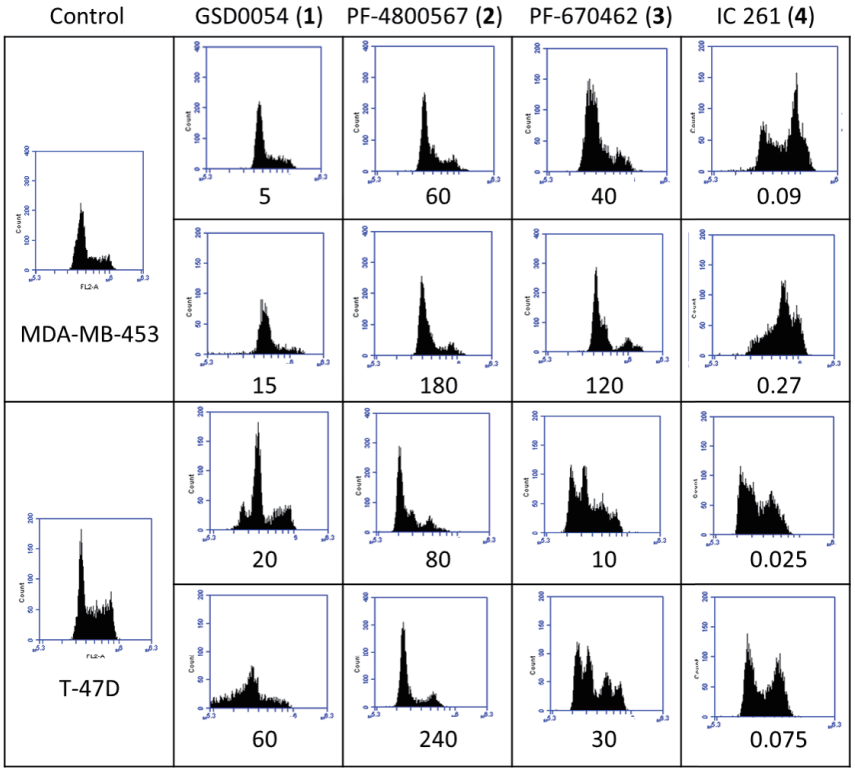

Fig. 4. Cell cycle histograms of MDA-MB-453 and T-47D untreated cells (control) and cells treated (drug concentration in μM) for 24 h with compounds 1-4 at the indicated doses.

4. Discussion

In our group, we seek rational approaches based on phenotypic changes induced in cells by bioactive compounds that may allow the identification of possible biological targets for any given small molecule. With this strategy, we have discovered the novel CK1ε selective inhibitor GSD0054 (1) [8]. CK1ε is a modulator of the Wnt/β-catenin signaling pathway and represents a potential target for developing anticancer drugs with high therapeutic index [7]. To the best of our knowledge, selective CK1ε inhibitors are scarce. One of the main drawbacks in the search for ICK1ε is that CK1δ holds $>$ 98% sequence identity with CK1ε. Thus, inhibitors in public databases in general are reported as dual ICK1δ/ε [11]. Considering the differences in biological involvement of both isoforms, the reports in the literature seem confusing and produce apparently contradictory results. In the current study, we performed some phenotypic studies on our ICK1ε GSD0054 (1) and compared the results with the established compounds ICK1ε PF-4800567 (2), and the ICK1δ/ε PF-670462 (3) IC 261 (4).

Initially, we found that CK1ε inhibitors 1-4 exerted anti-proliferative effects on MDA-MB-453 and T-47D cell lines (Table 1). Thus, selective CK1ε inhibitors (1-2) were more active against MDA-MB-453 (β-catenin negative) cells, whilst T-47D (β-catenin positive) cells were more sensitive to the dual CK1δ/ε inhibitors (3-4). The discouraging outcome of this result is that all compounds were able to induce growth inhibition independently of the Wnt/β-catenin signaling pathway. In this particular context, it was found that compound 2 did not exert anti-proliferative effects on human cell lines HEK293 and HT1080 after 7 days of exposure at 1 μM [7]. Our results demonstrate that larger doses of 2 are necessary to observe growth inhibition. At this point, we wondered if the differences in potency observed for compounds 1-4 could be related to other factors. We next explored the ability of P-gp to reduce the antiproliferative activity of compounds 1-4. This transmembrane efflux transporter was initially characterized as overexpressed in tumor cells exhibiting multiple drug resistance to a wide spectrum of structurally-unrelated anticancer drugs [12]. None of the tested compounds was affected by the overexpression of P-gp (Table 3).

When the expression of β-catenin in T-47D cells was studied, only compounds 1 and 4 induced a downregulation of this protein after 24 hours of treatment, in a dose-dependent manner (Fig. 3). Our findings do not agree with Cheong et al. [7], who, based on the data obtained from the comparison between compounds 2 and 4 proposed that the combined inhibition of CK1δ/ε was more effective in promoting inhibition of Wnt/β-catenin signaling and cell proliferation. Our data suggest that either compounds 2 and 3 do not inhibit CK1ε and subsequently β-catenin activity in these cell lines, or that CK1ε inhibition might have other consequences distinct of Wnt/β-catenin inhibition that are also able to inhibit growth. Similar results were found by Rodriguez et al. [13] when they observed that suppression of CK1ε expression using shRNA constructs led to an antiproliferative effect in ovarian cancer cells, which was independent of Wnt/β-catenin activity. Therefore, it is possible that inhibition of CK1ε interferes with cell growth without affecting β-catenin expression, partially explaining the profiles observed for compounds 2 and 3.

To further understand the similarities and differences in the mechanism of action of the four inhibitors, we proceeded with cell

cycle analysis by flow cytometry. Interestingly, we observed that only compound 1 was able to produce cell death in T-47D cells as deduced from the increase in the sub-G_1 population (Fig. 4). This effect was not observed for MDA-MB-453 cells, marking a difference in the response between these cell lines after exposure to compound 1. However, the effect of inhibitors 2-4 only induced an accumulation of cells at different cell cycle phases. Our data partially support the findings of Cheong et al. [7], who described that compounds 2 and 3 were unable to induce cancer cell death. In addition, compound 4 was found to bind to β-tubulin with an affinity similar to colchicine that is a potent inhibitor of microtubule polymerization [7]. This is consistent with our cell cycle results, where inhibitor 4 induced accumulation of cells at G_2/M phase. Similarly, treatment of primary mouse embryo fibroblasts with compound 4 caused an accumulation of cells in G_2/M phases of the cell cycle, but it was not able to produce cancer cell killing [14].

As aforesaid, compound 1 was able to produce selective cell death in T47D cells which express β-catenin, where the sub-G_1 population accounted for 41% after exposure to a 60 μM concentration ($\approx 3\times$ GI_{50}). In contrast, the sub-G_1 population was only 6% in cells lacking active β-catenin (MDA-MB-453) after exposure to 15 μM of compound 1 ($\approx 5\times$ GI_{50}). This result is consistent with previous findings that selective cell killing may be occurring when Wnt signaling is known to be aberrantly activated [15]. When considering ROS production (Fig. 5), MDA-MB-453 cells were not affected by compound treatment. This is consistent with the cell cycle experiments. However, the effect on T-47D cells cannot be correlated with the cell cycle experiments, except for our ICK1ε GSD0054 (1).

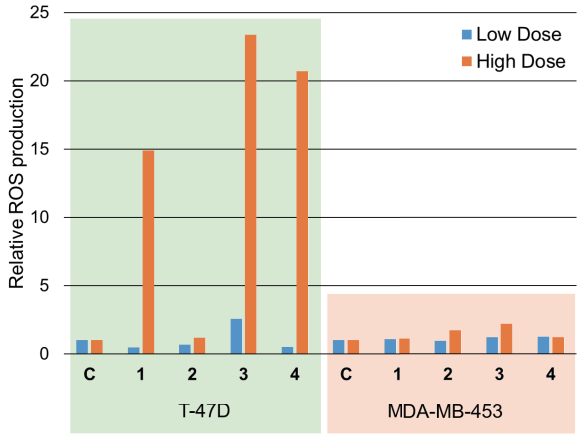

Fig. 5. ROS production increase in MDA-MB-453 and T-47D cells treated with drug (dose in μM) for 48 h with compounds 1-4 at the indicated concentrations. LD (low dose) of 1, 10, 10, 0.1 and HD (high dose) of 10, 100, 100, 1 μM for 1-4, respectively.

The docking results showed discrepancies with the antiproliferative data. Thus, the best docking score does not assure an actual superior cell growth inhibitory activity. In this context, although compound 2 displayed the most favourable interaction with CK1ε, it turns out to have the least potent antiproliferative activity, a null effect on β-catenin expression, no cell killing and lack of ROS production. One could argue that the docking results for this compound are biased towards more favourable outcome. This compound was co-crystallized *in silico* with the protein structure that we used in our docking calculations and therefore the protein is in a perfect conformation to accommodate it, producing better docking scores. However, our lead compound 1 having a docking score of −5.8 kcal/mol was able to: (a) inhibit cell proliferation, (b) inhibit β-catenin expression in T-47D cells, (c) induce ROS production and also (d) induce cell killing as observed with the accumulation of cells in the sub-G_1 phase.

In summary, we have performed a preliminary study of the mechanism of action of GDS0054 (1) in MDA-MB-453 and T-47D cells. We observed that our lead compound exhibits antiproliferative activity against MDA-MB-453 and T-47D cells despite the fact that MDA-MB-453 cells do not have active β-catenin. Cell cycle studies indicated that selective cell killing occurred in the more resistant, but β-catenin active, tumor cell line T-47D. Furthermore, GSD0054 (1) was not a transport substrate of P-gp. The comparative study with other three known CK1ε inhibitors revealed notable differences among the mechanism of action of all compounds.

Taken collectively, the results indicate that the mechanism of action might differ depending on the tumor cell line; it is possible that it affects different intracellular pathways or that the anti-proliferative effect is independent of β-catenin function. Further mechanistic studies are required to understand the exact role of GSD0054 (1) and its potential use as a therapeutic agent against cancer.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Vielhaber E, Virshup DM. Casein kinase I: from obscurity to center stage. *IUBMB Life*, 2001; 51: 73-78.

[2] Knippschild U, Wolff S, Giamas G, Brockschmidt C, Wittau M, Würl PU, Eismann T, Stöter M. The role of the casein kinase 1 (CK1) family in different signaling pathways linked to cancer development. *Onkologie*, 2005; 28: 508-514.

[3] Knippschild U, Krüger M, Richter J, Xu P, García-Reyes B, Peifer C, Halekotte J, Bakulev V, Bischof J. The CK1 family: contribution to cellular stress response and its role in carcinogenesis. *Front Oncol*, 2014; 4: 96.

[4] Yang WS, Stockwell BR. Inhibition of casein kinase 1-epsilon induces cancer-cell-selective, PERIOD2-dependent growth arrest. *Genome Biol*, 2008; 9: R92.

[5] Price MA. CK1, there's more than one: casein kinase I family members in Wnt and Hedgehog signalling. *Genes Dev*, 2006; 20: 399-410.

[6] Kim SY, Dunn IF, Firestein R, Gupta P, Wardwell L, Repich K, Schinzel AC, Wittner B, Silver SJ, Root DE, Boehm JS, Ramaswamy S, Lander ES, Hahn WC. CK1ε is required for breast cancers dependent on β-catenin activity. *PLoS One*, 2010; 5: e8979.

[7] Cheong JK, Nguyen TH, Wang H, Tan P, Voorhoeve PM, Lee SH, Virshup DM. IC261 induces cell cycle arrest and apoptosis of human cancer cells via CK1δ/ε and Wnt/β-catenin independent inhibition of mitotic spindle formation. *Oncogene*, 2011; 30: 2558-2569.

[8] Silveira-Dorta G, Sousa IJ, Fernandes MX, Martín VS, Padrón JM. Synthesis and identification of unprecedented selective inhibitors of CK1ε. *Eur J Med Chem*, 2015; 96: 308-317.

[9] Silveira-Dorta G, Donadel OJ, Martín VS, Padrón JM. Direct stereoselective synthesis of enantiomerically pure *anti*-β-amino alcohols. *J Org Chem*, 2014; 79: 6775-6782.

[10] Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, Boyd M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst*, 1991; 83: 757-766.

[11] Gilson MK, Liu T, Baitaluk M, Nicola G, Hwang L, Chong J. BindingDB in 2015: A public database for medicinal chemistry, computational chemistry and systems pharmacology. *Nucleic Acids Res*, 2016; 44: D0145-D1053.

[12] Bradley G, Juranka PF, Ling V. Mechanism of multidrug resistance. *Biochim Biophys Acta*, 1988; 948: 87-128.

[13] Rodriguez N, Yang J, Hasselblatt K, Liu S, Zhou Y, Rauh-Hain JA, Ng SK, Choi PW, Fong WP, Agar NY, Welch WR, Berkowitz RS, Ng SW. Casein kinase 1 epsilon interacts with mitochondrial proteins for the growth and survival of human ovarian cancer cells. *EMBO Mol Med*, 2012; 4: 952-963.

[14] Behrend L, Milne DM, Stöter M, Deppert W, Campbell LE, Meek DW, Knippschild U. IC261, a specific inhibitor of the protein kinases casein kinase 1-delta and -epsilon, triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. *Oncogene*, 2000; 19: 5303-5313.

[15] Hao J, Ao A, Zhou L, Murphy CK, Frist AY, Keel JJ, Thorne CA, Kim K, Lee E, Hong CC. Selective small molecule targeting β-catenin function discovered by in vivo chemical genetic screen. *Cell Rep*, 2013; 4: 898-904.