New Biological Evaluation of Thienoquinolines as Disruptors of the PKCε/RACK2 Protein–Protein Interaction

G. B. Lapa^{*a*}, * (ORCID: 0000-0001-5650-2470), P. Gruber^{*b*}, G. Untergasser^{*c*}, N. I. Moiseeva^{*d*}, and J. Hofmann^{*b*}

^a Pirogov Medical University, Moscow, 117997 Russia

^b Biocenter, Division of Medical Biochemistry, Innsbruck Medical University, Innsbruck, A-6020 Austria

^c Laboratory of Experimental Oncology, Tyrolean Cancer Research Institute, Innsbruck, A-6020 Austria

^d Blokhin National Medical Research Center of Oncology, Moscow, 115478 Russia

*e-mail: lapa.gb@yandex.ru

Received September 5, 2022; revised October 12, 2022; accepted October 14, 2022

Abstract—The superfamily of the protein kinase C (PKC) comprises ten isozymes and is widely known for its key role in signal transduction. Protein kinase C ϵ (PKC ϵ) is known to play key roles in tumor suppression. PKCE requires activation to interact with RACK2, and the adaptor protein then translocates activated PKCE to subcellular sites within the proximity of their substrates. An EAVSLKPT peptide interferes with the interaction of PKCE and its adaptor protein RACK2. Since signaling in the malignant cells are sufficiently changed then the scope and limitations of PKCe as a anticancer drug target has to be estimated more clearly. Acquiring isozyme-selective inhibitors is a difficult task due to the high sequence similarity within the ten PKCs. Small molecule-disruptors of the PKCe/RACK2 protein–protein interaction could suppress PKCe signaling and reduce malignant properties. The EAVSLKPT peptide was used as a base of a pharmacophore model. Thieno[2,3-b]quinolines as a wide cluster of specific small-molecule inhibitors of the PKCe/RACK2 protein– protein interaction and PKCe signaling were revealed. The structural features of active thieno[2,3-b]quinolines were expanded on the basis of this pharmacophore model. The interaction between PKCe and RACK2 was measured using an ELISA-based assay. It was found that N-(4-acetylphenyl)-3-amino-6,7-ethelendioxythieno[2,3-b]quinoline-2-carboxamide (1b) shows promising inhibitory activities on the interaction of PKCe with its adaptor protein, the receptor for activated C-kinase 2 (RACK2), hence interfering with PKCe signaling. Both 1a and 1b did not show some cytotoxic properties on susceptible PC-3 cell line but both active compounds showed a significant antisprouting activity. The quinolines without thiophene ring as "open" analogs of 1b were inactive in primary assays. A structural isomer of (1a meta-acetyl), compound (1b para-acetyl) was found to exhibit, in addition to strong inhibitory activity on PKC ϵ signaling with an IC₅₀ of 4.25 μ M, also anti-angiogenic activities. Thus thieno[2,3-b]quinolines 1a and 1b could be reliable and selective biochemical tools to investigate of PKCe/RACK2 effects.

Keywords: protein–protein interaction, PKCε, RACK2, PKCε inhibition, angiogenesis inhibitor, metastasis inhibitor, thienoquinoline, thieno[2,3-b]quinoline, pharmacophore **DOI:** 10.3103/S0027131422070082

INTRODUCTION

The protein kinase C (PKC) family of enzymes can be classified into three groups of isozymes: the conventional (α , β 1, β 2), novel (δ , ϵ , η , θ) and atypical (ι , ζ). Protein kinase C ϵ (PKC ϵ) is known to play key roles in tumor suppression [1, 2].

Cardiac hypertrophy [3], protection from ischemic insult [3], nociceptor function [4], macrophage activation [5], diabetes [6] and in alcohol consumption

[7]. Hence, selective inhibitors of PKC ϵ will be valuable tools for investigating the physiological function of this isozyme and are expected to have pharmacological potential for the prevention and treatment of cancer, strokes, drug addiction or pain [8–10].

The human genome encodes approximately 500 kinases, and the different kinase families share a well-conserved ATP binding site [11, 12]. This renders the design of selective competitive inhibitors of ATP a very challenging task. Rather than targeting the ATP binding site of PKC ε , the EAVSLKPT peptide interferes with the interaction of PKC ε and its adaptor protein, the receptor for activated C-kinase 2 (RACK2 or

Abbreviations: Basic fibroblast growth factor, bFGF; primary human umbilical vein endothelial cells, HUVEC; protein kinase C, PKC; protein kinase B, PKB; receptor for activated C-kinase 2, RACK2; vascular endothelial growth factor, VEGF.

 β COP) [13, 14]. PKC ϵ requires activation to interact with RACK2, and the adaptor protein then translocates activated PKC ϵ to subcellular sites within the proximity of their substrates [15, 16].

Recently we reported on the discovery of thieno[2,3-b]quinolines as a novel cluster of specific small-molecule inhibitors of PKC ε signaling [17, 18]. The most active compound identified as *N*-(3-acetyl-phenyl)-9-amino-2,3-dihydro-1,4-dioxino[2,3-g]thieno[-2,3-b]quinoline-8-carboxamide (1a; PKCe141; IC₅₀ 5.9 μ M; Table 1) on the base of a pharmacophore model [17, 18]. It was shown previously that the **A** and **B** fragments demonstrated a robust effect on the activity (Scheme 1) [17, 18]. Since the meta-isomer 1a demonstrated high activity the previously discussed pharmacophore model could be expanded to the paraisomer 1b (Scheme 2). It was synthesized [17, 18]. The small library of 1c-1s was purchased on the base of

this model (Table 1). This expansion could be used to propose some activity for the compounds with the para-substituents of big size or thioethers as the compounds with an "open" form of the thiophene ring. Long flexible linkers of thioethers could confirm the importance of both, the amino group and the distance between the ethylenedioxy ring and the acetyl group to provide high bioactivity (Scheme 2).



Scheme 1.



Scheme 2. Pharmacophore model derived from EAVSLKPT (Short peptide was extracted from the crystal structure of the PKCε protein, PDB code 1GMI). Hydrogen bonding and hydrophobic features are indicated with green and brown spheres, respectively. Alignment of **1b** with the pharmacophore model was generated using Discovery Studio 3.5. (Accelrys)

In this follow-up study we further investigated the biochemical and pharmacological properties of thienoquinolines as disruptors of the PKC ϵ /RACK2 protein—protein interaction and, in addition, expanded our investigations to thioethers **2** as "open" analogs of **1** in order to obtain a more complete picture of the biological properties and structure-activity relationships (SAR) of this class of compounds (Table 1). Additionally, we report SAR for new thieno[2,3b]quinolines and new data of a further biological evaluation of **1b** as the most active compound.

EXPERIMENTAL

Chemistry

Chemical synthesis of all compounds was described previously and repeated in supplementary materials for convenience [17]. The improved synthetic procedure for final compounds (1, 2) is described below (Scheme 3). The purity of the synthetic compounds was approved as a single spot on TLC at UV 254 nm (Merck TLC plate, DCM-Methanol). Original ¹H-NMR and ESI-MS spectra are collected in supplementary material.

Table 1. St	ructure and i	nhibition of P	$KC\epsilon/RACK2$	interaction assav	in vitro for 1 ar	id 2

#	Vendor ID	RI	R2	R3	N ^a	Concentration, μM	% PKCɛ\RACK2 interaction ^b	SD	SEM
1a*	ASN05545158 PKCe141	-0-CH ₂ -	-CH ₂ -O-		48	10	23.6	10.2	1.5
1a*	_	-	-	_	80	25	13.3	6.1	0.7
1b*	PKCe2138	-0-CH ₂ -	-CH ₂ -O-	······O	12	10	22.8	5.8	1.7
1b*	_	-	-	_	8	25	11.0	1.5	0.5
1c	ASN05545085	-0-CH ₂ -	-CH ₂ -O-		12	25	25.9	7.8	2.0
1d	ASN05544844	Н	-O-CH ₃		12	25	27.8	7.0	1.7
1e	ASN05545061	-0-CH ₂ -	-CH ₂ -O-	rover the second s	12	25	28.5	6.0	1.5
1f	ASN05545126	-0-CH ₂ -	-CH ₂ -O-	0 0	12	25	40.5	12.9	3.2
1g	ASN05544732	-O-CH ₃	Н		12	25	42.2	9.4	2.3
2a	_	-0-CH ₂ -	-CH ₂ -O-		8	10	83.8	19.1	6.8

^{*} Ref. [17]. Vendor is Asinex Ltd. (NC, USA), ^a N-number of tests performed, ^b % PKC ϵ /RACK2 interaction = The percentage of PKC ϵ /RACK2 interaction with the indicated concentration of the compound compared with untreated controls. SD-standard deviation, SEM—the standard error of the mean.



Synthesis of 1a, 1b and 2a Scheme 3. Synthesis of 1a, 1b and 2a

3-Amino-thieno[2,3-b]quinoline-2-carboxylamids (7). A mixture of **3** (1 eq), respective halocompound (1.25 eq) and sodium methylate (3 eq) in abs. methanol was refluxed for 1.5 hr. The formed solid was collected by filtration, washed several times with methanol and finally with water and recrystallized from DMF-methanol 7:3.

In this way the following compounds were prepared:

1a ¹H-NMR (500 MHz, dmso-d6) 2.53 (s, 3H), 4.42 (m, 4H), 7.45 (m, 3H), 7.55 (s, 2H), 7.67 (d, 1H, J 10Hz), 8.01 (d, 1H, J 10Hz), 8.34 (s, 1H), 8.89 (s, 1H), 9.58 (s, 1H). MW Calc. for $C_{22}H_{17}N_3O_4S$ 419.0939. Found in ESI-MS 420.1043 (M+H). m.p. > 250°C. **1b** ¹H-NMR (500 MHz, dmso-d6) δ : 2.53 (3H, s, CH3), 4.41 (1H, dd, J 3, 3, -CH2-CH2-), 7.44 (2H, s, H-5, H-8), 7.62 (2H, s, NH2), 7.91 (4H, dd, J 3, 3, arom), 8.89 (1H, s, H-4), 9.68 (1H, s, HN-C=O). MW Calc. for $C_{22}H_{17}N_3O_4S$ 419.0938. Found in ESIms 420.1047 (M+H) 100%. m.p. > 250°C. (Original ¹H-NMR, ESI-MS are in Supplementary material – **1a** – H141, *PKCe141*, **1b** – *LCTA2138*, *PKCe2138*).

2-[(3-cyanoquinolin-2-yl)thio]-N-phenylacetamide (2a), A mixture of 3 (1 eq), respective halocompound (1.25 eq) and fused sodium acetate (3 eq) in abs. ethanol was refluxed for 2 h. The formed solid was collected by filtration, washed several times with methanol-water 3:1 and recrystallized from DMF-methanol 1:1. In this way the following compounds were prepared:

2a, ¹H-NMR (500 MHz, dmso-d6): 2.54 (s, 3H), 4.25 (s, 2H), 4.36 (m, 4H), 7.23 (s, 1H), 7.40 (s, 1H), 7.47 (t, 1H, J 5Hz), 7.65 (d, 1H, J 5Hz), 7.65 (d, 1H, J 5Hz) 8.20 (s, 1H), 8.69 (s, 1H), 10.64 (s, 1H). MW Calc. for $C_{22}H_{17}N_3O_4S$ 419.0939. Found in ESI-MS 420.1121 (M + H). (Original ¹H-NMR, ESI-MS are in Supplementary material—*LCTA2022 - 20*).

Biochemistry

Solutions. 25 mM stock solutions of all compounds were prepared in DMSO or DMF. These stock solu-

tions were incubated at 56°C for 1 h to dissolve the compounds completely.

Cells. For cell proliferation PC-3 cells were seeded at ~10000 cells per well in 96-well plates. After 4 h various concentrations of 1a, 1b were added and left for 72 h. Cell proliferation was determined by the SRBassay. Human endothelial cells from different donors (HUVECs, n = 3) were purchased from Promocell. Human mammary epithelial cells (HMECs) were cultivated in Endothelial Cell Growth Medium (EGM2) with recommended supplements (all from Promocell) on collagen-type-I (Sigma Biochemicals) coated ventilated plastic flasks. Cells were passaged by the cell detach kit (Promocell) consisting of 30 mM Hepes, 0.04/0.03% Trypsin/EDTA Solution and Trypsin Neutralizing Solution (TNS) and analyzed for the expression of PECAM1 (FITC-labeled mouse-antihuman CD31 mab WM59, BD Biosciences) and VEGFR2 (PE-labeled mouse anti-human KDR, clone #89106, R&D systems) by flow cytometry.

PKCe/RACK2 in vitro Binding Assay

The PKC ε /RACK2 in vitro binding assay was run as detailed in [17].

Recombinant RACK2 tagged with maltose-binding protein (RACK2-MBP) was purified on columns with amylose-resin (New England Biolabs, Ipswich, MA) The interaction between PKCe and RACK2 was measured using an ELISA-based assay. The 96-well EIA/RIA high binding plates (Costar) were coated with 100 ng of recombinant PKCe (ProQinase, Freiburg, Germany) in buffer A (20 mM Tris-HCl/100 mM NaCl, pH 7.5) at 4°C on a shaker with gentle agitation overnight. The plate was washed twice with $225\,\mu$ L/well buffer A. After blocking of unspecific bindings it with 225 µL of sterile-filtered 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in buffer A at room temperature for 3 h, the plate was washed twice with 225 µL of this buffer. PKCE was left untreated or activated by addition of 60 µg/mL phosphatidylserine (Sigma-Aldrich) and 100 nM TPA (Sigma-Aldrich) in a volume of 50 µL of buffer A for 10 min at 30°C. Recombinant purified RACK2-MBP (obtained from Prof. Dr. Daria Mochly-Rosen, Stanford University, USA) was either left untreated or incubated with the indicated compound at room temperature for 30 min in a final volume of 50 μ L of buffer A. Then 500 ng RACK2-MBP was added to untreated or activated PKC ε for 1 h at room temperature for binding. The plate was washed twice with 225 µL of buffer A. Subsequently, 100 µL of RACK2-specific rabbit anti-RACK2 polyclonal antibody (obtained from Prof. F. Wieland, University of Heidelberg, Germany, diluted 1: 20000 in 3% BSA/buffer A) was added for 1 h at room temperature. The plate was then washed three times with $225 \,\mu\text{L}$ of buffer A, and a goat anti-rabbit HRP-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1: 20000 in 100 µL of 3% BSA/buffer A) was added for 1 h at room temperature. After three washes with 225 μ L of buffer A, 100 μ L of ABTS substrate (0.5 mg/mL) diluted in ABTS buffer (Roche, Vienna, Austria) was added and the plate was incubated in the dark for 30–180 min. Color development was measured on a plate reader at a wavelength of 420 nm.

Cell proliferation and migration assays. Real time cell proliferation and migration experiments were performed using the RTCA DP instrument (Roche Diagnostics GmbH), which was placed in a humidified incubator maintained at a 5% CO₂ at 37°C. For proliferation assays, cells were seeded in complete medium in 16-well plates (E-plate 16, Roche Diagnostics GmbH) at a density of 5000 cells/well after coating with 10 μ g/mm² fibronectin (Sigma Biochemicals). The plate containing gold microelectrodes on its bottom was monitored every 10 min for 4 h (adhesion process), then once every 30 min, until the end of experiment, which was in total 72 h (cell proliferation). Cell migration was performed using special 16well plates with 8 µm pores (CIM-plate 16, Roche Diagnostics GmbH). These plates, resembling conventional trans wells, have microelectrodes placed on the bottom side of the membrane. Cells were seeded into the upper chamber at a density of 30000 cells/well in serum-free medium; the lower chamber was filled with complete medium. The plate was monitored every 15 min for 12 h. Data analysis was performed using RTCA software 1.2 supplied with the instrument.

Spheroid sprouting assay. HUVEC spheroids where generated overnight in hanging-drop culture consisting of 400 cells in EBM-2 medium, 2% FCS and 20% methylcellulose (Sigma Biochemicals). Spheroids were embedded in collagen type I from rat tail (Becton Dickinson) and stimulated with 50 ng/mL VEGF (Sigma Biochemicals) in the presence or absence of compounds or control substances (DMF, Bortezomib). Sprouts were also analyzed by inverted transmission-microscopy (Zeiss Axiovert 200 M) and documented by a digital imaging (Axiovision Software, Zeiss). The cumulative sprout length (CSL) was analyzed after printing of high quality pictures and counting by two independent blinded observers.

 IC_{50} determination and statistics. IC_{50} values were determined with CalcuSyn (Biosoft, Cambridge, UK).

RESULTS AND DISCUSSION

Short description of the synthesis of 1a, 1b and 2 are reported in Scheme 3. Briefly, the thioamides 3 and the final compounds were synthesized according to a well-developed procedure [17–21]. Alkylation of the sulfur of the thioamides 3 was only observed in the presence of a weak base such as sodium acetate to gain the thioethers 2a. Strong bases such as a sodium methylate facilitated both S-alkylation and cyclization of the nitriles 2 to produce the amides 1 [17, 21]. All details of this synthesis are in the supporting materials. Scaling up the procedures in order to synthesize several grams of the most interesting compounds 1a, 1b for a more thorough biological evaluation was straightforward. A library of compounds (1c-1f) was purchased.

It was found that the para-isomer **1b** was more soluble than meta-isomer **1a** (6.7 mg of **1a** and 10.1 mg of **1b** are soluble in 1.0 ml DMSO at 25°C, respectively).

Table 1 contains the structures of the several, most active, tested compounds and measured by an ELISAbased assay data of inhibition PKC ϵ \RACK2 interaction. SAR clearly indicates that the 3-amino group and the ethylenedioxy ring are the most important fragments for the more active compounds (**1a-c**) (Table 1 and Scheme 2). The substituents of big size (**1c**) did not show any preferences. Small substituents (**1e**) and heterocycles (**1c**) did not show enough acceptor properties (Scheme 2 and Table 1). Thioether **2** was less active in comparison with thieno[2,3-b]quinolines (**1a-1g**) (Table 1). Obviously the set of A-C fragments (scheme 1) for **1a**, **1b** is optimal for robust inhibition of PKC ϵ -signaling. All data of SAR are given in supplementary materials.

A PKC ϵ /RACK2 binding assay was used as the primary in vitro approach for evaluating the biological activity of these molecules. This assay has been described in detail previously [17]. Briefly, RACK2 was bound to activated PKC ϵ in vitro. The assay measures the disruption of the PKC ϵ /RACK2 interaction by small organic molecules. Among all compounds tested, isomers **1a** and **1b** were identified as most active (Table 1), with IC₅₀ values of 5.9 and 4.25 μ M, respectively. These values are within the activity range of the peptide inhibitor EAVSLKPT, which has an IC₅₀ of 1.02 μ M [16, 17]. The dose-dependent activity of **1a** and **1b** is reported in Fig. 1.

An important question is whether these compounds inhibit PKC ϵ or other PKC isozymes directly. As shown in Fig. 2, **1b** did not inhibit the PKC isozymes α , $\beta 2$, ι , ζ significantly. There are around 500 kinases in a cell [12]. In our previous study, a test of **1a**



Fig. 1. Effects of compounds 1 and 1c on PKC ϵ /RACK2 interaction in vitro. Both compounds prevent the interaction of PKC ϵ with RACK2 in a dose-dependent manner. The means (+/– SD) of two independent experiments in which four samples were taken within each experiment are shown.

on a panel of 109 different kinases showed inhibition of some of these enzymes [17].

Since selected compounds were not intended to inhibit PKCE directly or to inhibit other kinases then for the current work we also submitted 1b for testing in this kinase panel. The majority of these kinases are not inhibited by 1b at 50 µM concentration, and those which are, are less affected than by 1a (Table 2). An exception is protein kinase B-beta (also known as AKT2 or PKB- β), which is inhibited more strongly by compound **1b** at the high concentration of 50 μ M. Why and how **1b** inhibits PKB- β rather well is not known at present. This kinase plays a key role in multiple cellular processes such as apoptosis, transcription, cell proliferation and migration. For the inhibition of kinases shown in Table 2, 50 µM of the compounds were employed. However, a 50 µM concentration might not be used usually in vivo because the IC₅₀ is $4.24 \,\mu$ M. Overall, in case of pharmacological use or in vivo tests the lower inhibition of kinases in general could indicate that fewer side effects may be expected from 1b. This would mean that in a



Fig. 2. Effects of 50 μ M compound 1b on PKC isozymes. The method is described in Ref. 18. Three independent experiments in which 4 samples were taken within each experiment are shown (+/- SD).

MOSCOW UNIVERSITY CHEMISTRY BULLETIN Vol. 77 Suppl. 1

potential pharmacological use of **1b**, kinase inhibition might not play a role.

PKC ε is thought to be involved in prostate cancer [8, 22]. Therefore, inhibition of cell proliferation by **1b** was evaluated on PC-3 prostate carcinoma cells which express PKC ε as shown in Fig. 3. Even at 50 µM concentration **1b** did not inhibit cell proliferation in this cell type. It has been shown previously that PKC ε does not increase cell proliferation of HeLa cervix carcinoma cells [23]. These are indications that suppression of PKC ε -activity does not inhibit cell proliferation and support studies showing that PKC ε is a tumor suppressor [1].



Fig. 3. Effect of compound 1b on cell proliferation. ~10000 PC-3 cells in 200 μ L were seeded per well in 96-well plates. After 4 h the indicated concentrations of compound 1b were added and left for 72 h. Cell proliferation was determined by the SRB-assay. The means (+/– SD) of two different experiments in which four samples within each experiment are shown.

2022

#	Kinase	1a	SD, %	1b	SD, %
1	BTK	10.0	2.0	37.6	7.2
2	ERK1	27.0	6.0	64.5	6.1
3	МАРКАР-КЗ	40.0	1.0	27.8	1.4
4	PIM3	26.0	3.0	39.1	2.4
5	PKB-beta	39.0	3.0	13.6	0.1
6	RSK2	18.0	1.0	42.1	3.6

Table 2. Profile of kinase inhibition by 50 μ M solution of **1a**, **1b**. * (% of control, mean of two experiments)

* 109 different protein kinases were tested for their inhibition by **1a**, **1b** (see supporting materials). Only kinases sufficiently inhibited by compound **1a**, **1b** are shown. Screening was performed by the National Centre for Protein Kinase Profiling, Division of Signal Transduction Therapy, University of Dundee. The data is portrayed as mean % activity and standard deviation of assay duplicates. Inhibition of several kinases by **1a** is shown in reference [17].

Compounds **1a** and **1b** were selected for DTP NCI 60 cell line assay to prove our MTT results [24]. Both compounds did not show any significant results as cytotoxic agents. All results are in supporting materials. It worth to notice that in DTP NCI tests just **1b** revealed moderate suppression of A498 cell line growth only. Indeed we found that compound **1b** was

more potent with IC_{50} value of 31 μ M when **1a** was completely inactive with IC_{50} value > 100 μ M. Perhaps this moderate activity of **1b** depends on elevated AKT/mTOR signaling in A498 cells [25].

It has been shown previously that PKCE does not increase cell proliferation of HeLa cervix carcinoma



Fig. 4. Real-time measurement of endothelial cell proliferation and migration in the xCelligene system (a) Proliferation of HUVEC cells was analyzed on fibronectin-coated E-plates in triplicates over a time window of 36 h (mean \pm SEM) in direct comparison to respective controls (0.1% solvent dimethylformamide = untreated). (b) Migration of HUVECs was analyzed in triplicates on fibronectin-coated CIM-plates over a time window of 24 h (mean \pm SEM) in direct comparison to respective controls (0.1% dimethylformamide = untreated).



Fig. 5. Analysis of angiogenic sprouting of human endothelial cells (HUVEC) in vitro. Angiogenic sprouting of human endothelial cell spheroids in collagen-type-I/methylcellulose gel was analyzed after stimulation with 50 ng/mL human VEGF (mean \pm SEM). Control cells with VEGF stimulation displayed the maximal cumulative sprout length and served as positive control. The proteasome inhibitor bortezomib, a strong antiangiogenic drug, served as negative control to inhibit angiogenic sprouting and cumulative sprout length. Stars indicate *p* values < 0.05.

cells [23]. However, PKCE increases cell migration and is associated with metastatic spread and invasiveness of human cancer cells [8, 26]. It has also been reported that a selective PKCE agonist induces proangiogenic responses in endothelial cells, including formation of capillary like structures and cell growth [27]. Therefore, compounds 1a and 1b were tested on primary human umbilical vein endothelial cells (HUVEC – model of normal cells) in a real-time proliferation system (XCelligence, Roche Diagnostics) for effects on proliferation and cell numbers in growth medium containing 2% serum, bFGF and VEGF. Both compounds significantly inhibited HUVEC cell proliferation in a dose-dependent manner (Fig. 4a). HUVEC cell migration was assessed within 12 h after exposure to the compounds by real time-measurement (XCelligence, Roche Diagnostics) of migrating cells through fibronectin-coated pores (8 µm) and a gradient of 2% serum and VEGF. Only 1a slightly inhibited cell migration in vitro at 25 µM whereas **1b** had no impact on short time cell migration (Fig. 4b). These observations indicate that both compounds are acting on cell proliferation processes of HUVEC cells rather than on the migration of cells. Sprouting has been observed in relation with PKCE induction [27]. Since induction of PKCe-activity increases angiogenic sprouting, HUVEC cells can be used as model for sprouting. Therefore HUVEC cells were used as a model for the tests of blood vessel formation. The cells were cultured as spheroids in hanging drops for 24 h. In comparison to 0.1% dimethylformamide (control) both compounds were inhibiting angiogenic sprouting at the low concentrations of 2.5 μ M. At this concentration, compound 1b significantly reduced the angiogenic sprouting response of human endothelial cells (Fig. 5). Since angiogenic sprouting is a process relying on proliferation processes and influenced by a complex 3D extracellular matrix, compound **1b** seems to be more potent to inhibit these processes than the less soluble compound **1a**. Our results have shown that **1a** and **1b** as inhibitors of the activation of PKCe also inhibit angiogenic sprouting. As can be seen in Fig. 5, **1b** shows better performance compared with **1a** in this assay.

CONCLUSION

In conclusion, we synthesized and tested a number of derivatives of compound 1a which was shown recently to act as an inhibitor of PKCE signaling [17, 18]. We found that the para-isomer (1b) exhibits better inhibition of the PKCe/RACK2 interaction compared to 1a and is more soluble. Compounds 1a and **1b** inhibited the proliferation of HUVEC endothelial cells (normal cells) but did not inhibit PC-3 prostate cancer cells. Since 1a and 1b did not show significant efficacy in the inhibition of proliferation and migration of cells then we can probably conclude that both 1a and 1b did not affect several main functions of AKT2 (PKB-beta). Both compounds also inhibited angiogenic sprouting. Because PKCE is involved in nociceptor function, both compounds are interesting for analgesic purposes [4], alcohol consumption [7] and also for inhibition of angiogenesis. Thus thieno[2,3-b]quinoline 1b could be reliable and selective biochemical tool to investigate of PKCE/RACK2 effects. In spite of **1a** and **1b** have shown significant decrease only angiogenic sprouting activity the PKCe could be reliable target for diminish of both sprouting and migration activity of cancer cells.

ACKNOWLEDGMENTS

This work was done in commemoration and respect of Professor Maria N. Preobrazhenskaya.

FUNDING

This work was supported by the grants P16477-B12 and P25491-B21 from the Austrian Science Fund, grant Prokinase Research from the European Commission (European Union integrated project LSHB-CT-2004-503467) and grant GZ: UNI-0404/728 from the Tyrolean Science Fund.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.3103/S0027131422070082.

REFERENCES

- Antal, C.E., Hudson, A.M., Kang, E., Zanca, C., Wirth, C., Stephenson, N.L., Trotter, E. W., Gallegos, L.L., Miller, C.J., Furnari, F.B., Hunter, T., Brognard, J., and Newton, A.C., *Cell*, 2015, vol. 160, p. 489.
- Garg, R., Cooke, M., Benavides. F., Abba, M.C., Cicchini, M., Feldser, D.M., and Kazanietz, M.G., *Cancer Res.*, 2020, vol. 80, no. 23, p. 5166.
- Pass, J.M., Zheng. Y., Wead, W.B., Zhang, J., Li, R.C., Bolli, R., and Ping, P., *Am. J. Physiol.*, 2001, vol. 280, p. H946.
- Cesare, P., Dekker, L.V., Sardini, A., Parker, P.J., and McNaughton, P.A., *Neuron*, 1999, vol. 23, p. 617.
- Castrillo, A., Pennington, D.J., Otto, F., Parker, P.J., Owen, M.J., and Bosca, L., *J. Exp. Med.*, 2001, vol. 194, p. 1231.
- Ikeda, Y., Olsen, G.S., Ziv, E., Hansen, L.L., Busch, A.K., Hansen, B.F., Shafrir, E., and Mosthaf-Seedorf, L., *Diabetes*, 2001, vol. 50, p. 584.
- Hanim, A., Mohamed, I.N., Mohamed, R.M.P., Das, S., Nor, N.S.M., Harun, R.A., and Kumar, J., *Mini Rev. Med. Chem.*, 2020, vol. 20, no. 17, p. 1696.
- 8. Jain, K. and Basu, A., Cancers, 2014, vol. 6, p. 860.

- Shirai, Y., Adachi, N., and Saito, N., *FEBS J.*, 2008, vol. 275, p. 3988.
- 10. Akita Y., FEBS J., 2008, vol. 275, p. 3995.
- 11. Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S., *Science*, 2002, vol. 298, p. 1912.
- Fedorov, O., Marsden, B., Pogacic, V., Rellos, P., Muller, S., Bullock, A.N., Schwaller, J., Sundstrom, M., and Knapp, S., *Proc. Natl. Acad. Sci. U. S. A.*, 2007, vol. 104, p. 20523.
- Csukai, M., Chen, C.H., De Matteis, M.A., and Mochly-Rosen, D.J., *Biol. Chem.*, 1997, vol. 272, p. 29200.
- 14. Schechtman, D. and Mochly-Rosen, D., *Oncogene*, 2001, vol. 20, p. 6339.
- 15. Mochly-Rosen, D., Khaner, H., and Lopez, J., *Proc. Natl. Acad. Sci. U. S. A.*, 1991, vol. 88, p. 3997.
- Gray, M.O., Karliner, J.S., and Mochly-Rosen, D.J., *Biol. Chem.*, 1997, vol. 272, p. 30945.
- Rechfeld, F., Gruber, P., Kirchmair, J., Boehler, M., Hauser, N., Hechenberger, G., Garczarczyk, D., Lapa, G.B., Preobrazhenskaya, M.N., Goekjian, P., Langer, T., and Hofmann, J., *J. Med. Chem.*, 2014, vol. 57, p. 3235.
- Novel inhibitors of protein kinase c epsilon signaling, World Patent WO2014207213, 2014.
- Lapa, G.B., Bekker, O.B., Mirchink, E.P., Danilenko, V.N., and Preobrazhenskaya, M.N., *J Enzyme Inhib. Med. Chem.*, 2013, vol. 28, p. 1088.
- 20. Abdel-Rahman, A.E., Bakhite, E.A., Abdel-Moneam, M.I., and Mohamed, T.A., *Phosphorus, Sulfur Silicon Relat. Elem.*, 1992, p. 213.
- 21. Hafez, A.A.A., El-Dean, A.K., Hassan, A.A., El-Kashef, H.S., Rault, S., and Robba, M., *J. Heterocycl. Chem.*, 1996, vol. 33, p. 431.
- 22. Gorin, M.A. and Pan, Q.T., *Mol. Cancer*, 2009, vol. 8, p. 1.
- Garczarczyk, D., Toton, E., Biedermann, V., Rosivatz, E., Rechfeld, F., Rybczynska, M., and Hofmann J., *Cellular Signalling*, 2009, vol. 21, p. 745.
- Alley, M.C., Scudiero, D.A., Monks, P.A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R., *Cancer Res.*, 1988, vol. 48, p. 589.
- 25. Chhabra, R. and Nanjundan, M., *PLoS One*, 2020, vol. 15, no. 6, e0233887.
- 26. Stensman, H. and Larsson, C., *BMC Cancer*, 2008, vol. 8, 365, p. 1.
- Monti, M., Donnini, S., Morbidelli, L., Giachetti, A., Mochly-Rosen, D., Mignatti, P., and Ziche, M., *J Mol Cell Cardiology*, 2013, vol. 63, p. 107.