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Abstract Human kinases are one of the most promising targets for cancer therapy. Methods able to measure the effects of drugs on these cell agents remain crucial for biologists and medicinal chemists. The current work therefore sought to develop an in-capillary enzymatic assay based on capillary electrophoresis (CE) to evaluate the inhibition of phosphatidylinositol-3-kinase (PI3K), protein kinase B (Akt), and the mammalian target of rapamycin (mTOR). These kinases belong to the same signaling pathway PI3K/Akt/mTOR. For this proposal, the capillary was used as a nanoreactor in which a few nanoliters of the kinase, its substrate, adenosine triphosphate (ATP), and the potent inhibitor were separately injected. A transverse diffusion of laminar flow profiles (TDLFP) approach was employed to mix the reactants. Adenosine diphosphate (ADP) was detected online at 254 nm. The CE assay was first developed on the α isoform of PI3K. It was compared to five commercial kits frequently used to assess kinase inhibition, based on time-resolved fluorescence resonance energy transfer (TR-FRET) and bioluminescence. Each assay was evaluated in terms of sensitivity (S/B), reproducibility (Z'), and variability (r^2). This CE method was easily extended to assay the inhibition of the β, γ, and δ isoforms of PI3K, and of the other kinases of the pathway, Akt1 and mTOR, since it is based on in-capillary mixing by TDLFP and on ADP quantification by simple UV absorption. This work shows for the first time the evaluation of inhibitors of the kinases of the PI3K/Akt/mTOR pathway using a common in-capillary CE assay. Several inhibitors with a wide range of affinity toward these enzymes were tested.

Keywords Transverse diffusion of laminar flow profiles (TDLFP) · Mammalian target of rapamycin (mTOR) · Phosphatidylinositol-3-kinase (PI3K) · Protein kinase B (Akt)

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Introduction

The phosphatidylinositol-3-kinase (PI3K), the protein kinase B (Akt), and the mammalian target of rapamycin (mTOR) are the three main kinases constituting the PI3K/Akt/mTOR signaling pathway. This ubiquitous pathway regulates various cellular processes such as growth, angiogenesis, proliferation, and survival. It is frequently hyperactivated in cancer, and it plays an important role in tumor cell growth and survival. PI3K, the proximal kinase of the pathway, catalyzes the phosphoryl group transfer from adenosine triphosphate (ATP) to the 3′-OH position of its substrate, phosphatidylinositol-4,5-biphosphate (PIP_2). It thereby produces adenosine diphosphate (ADP) and phosphatidylinositol-3,4,5-triphosphate (PIP3) (Fig.S1.a, Electronic supplementary material), a secondary messenger that causes the recruitment of Akt, one of its major effectors (middle kinase of the pathway), on the plasma membrane. PI3K has received particular attention as it has been established that there is a high frequency of mutations in the gene encoding the p110α catalytic subunit of PI3K in human cancers. Four PI3K isoforms have been accounted for α, β, γ, and δ. Each isoform regulates specific signaling processes [1–3]. The other two enzymes of the

pathway are protein kinase B (Akt) and the mammalian target of rapamycin (mTOR) which are both Ser/Thr kinases. mTOR, located at the end of the pathway, belongs to the same family as PI3K, and some PI3K inhibitors are able to inhibit mTOR as well. Such dual inhibitors are very interesting drugs because they block the PI3K/Akt/mTOR pathway at two levels and prevent its reactivation due to the existence of regulatory feedback loops. Akt, also known as protein kinase B (PKB), is an essential regulator of several cellular signals including apoptosis, proliferation, and glucose uptake. The need to synthesize compounds that can inhibit all PI3K isoforms or specifically one of them as well as Akt and mTOR has been a great motivation for research teams around the world [4, 5]. Assaying the effect of inhibitors on these kinases has thus become very challenging. Providing cost- and time-effective methodologies to perform these tests is very important.

Capillary electrophoresis (CE) can be very promising in this field due to its rapidity, reproducibility, and consumption of only small sample volumes [6–10]. Several attempts have been made to develop enzymatic assays based on this analytical technique, particularly for measuring kinase activities [9, 11–15]. For example, extracellular signal-regulated kinases (ERK) were studied using CE with laser-induced fluorescence (LIF) [14] or UV (185 nm) [15] detection. CE was also employed to measure the dynamics of Akt in various cytokine-stimulated PC12 cells by monitoring the phosphorylation of its peptide substrate using UV detection at 190 nm [9]. However, CE has never been used to measure the IC_{50} of Akt inhibitors. Kinetic studies were carried out using CE-LIF to measure PI3Kα activity by following the phosphorylation of two fluorescent PIP_2 substrate derivatives. IC_{50} values of two known PI3K inhibitors, wortmannin and LY294002, were measured [16, 17]. In all these studies, the "pre-capillary" CE mode was used. For example, to conduct inhibition assays on PI3Kα, Huang et al. [16] incubated the reaction mixture (60 μL) for at least 90 min. The reaction was then quenched with an organic solvent before introducing 0.6 nl of the mixture in the capillary of the CE.

Recently, we developed [12] a simple and economic in-capillary kinase assay based on CE to screen inhibitors of four protein kinases: cyclin-dependent kinases (CDK1/cyclinB, CDK5/p25), glycogen synthase kinase 3-β (GSK3β), and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase (DYRK1A). This in-capillary enzymatic assay consisted in using the capillary of the CE as a nanoreactor in which a few nanoliters of reactants were separately and successively injected, and then mixed by transverse diffusion of laminar flow profiles (TDLFP) to initiate the enzymatic reaction. The ADP formed after a few minutes of incubation time was electrophoretically separated in the same capillary from the other reactants, online detected by UV at 254 nm, and quantified.

In-capillary CE based on TDLFP reactant mixing and simple UV detection of ADP has never been used to study signaling pathway kinases. The aim of this work was to develop an economic assay to assess the inhibition of the three main kinases of the PI3K/Akt/mTOR pathway using the in-capillary CE approach. For this purpose, a novel CE-based assay was first developed on the isoform α of PI3K and compared to five commercialized assay kits based on time-resolved fluorescence resonance energy transfer (TR-FRET) or bioluminescence. These kits are frequently used in the literature for kinase analysis [18–30] and are available in our laboratory. Assay performance was evaluated by testing three referenced PI3Kα inhibitors and by estimating their sensitivity (signal-to-background ration (S/B)), reproducibility (Z'), and robustness (r^2). Along the same lines, the CE assay was furthermore evaluated for the analysis of the three other isoforms of PI3K (β, γ, and δ). Several inhibitors with a wide range of affinity toward PI3K isoforms were tested in order to validate this assay. Finally, the CE assay was adapted and used to study the inhibition of the Akt1 isoform and mTOR.

Experimental section

Chemicals

Capillary electrophoresis

Ultrapure water (18 MΩ cm) was produced from an Elgastat apparatus (Elga, Villeurbanne, France). Dimethyl sulfoxide (DMSO), orthophosphoric acid, and sodium hydroxide were purchased from Fluka (Saint-Quentin-Fallavier, France). ADP, ATP, $MgCl_2$, poly(diallyldimethylammonium chloride) (PDADMAC 20 % *w*/*v*), PIP_2, and α,α,α-tris-(hydroxymethyl)-methylamin were purchased from Sigma-Aldrich (Lyon, France). FRAP1/mTOR and isoform PI3 kinase p110α/p85α (565 nmol/min/mg) were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). The inhibitors wortmannin, PI-103, and LY294002 (Fig. 1) were acquired from Upstate (Millipore, Billerica, MA, USA), Cayman Chemical (Ann Arbor, MI, USA), and Invitrogen (Life Technologies), respectively. The other inhibitors BEZ235, BYL719, GSK69063, MK-2206, and perifosine were purchased from Selleckchem (Souffelweyersheim, France). Active Akt/PKBα (or Akt1), crosstide, mTOR substrate, PI3 kinase isoforms (p110β/p85α, 246 nmol/min/mg; p120γ, 23 nmol/min/mg; and p110δ/p85α, 348 nmol/min/mg), syringes, and hydrophilic polyvinylidene fluoride (PVDF) Millex-HV Syringe Filters (pore size, 0.45 μm) were purchased from Millipore (Molsheim, France). Compounds 4 and 5 (Fig. 1) were home-made as reported elsewhere [20].

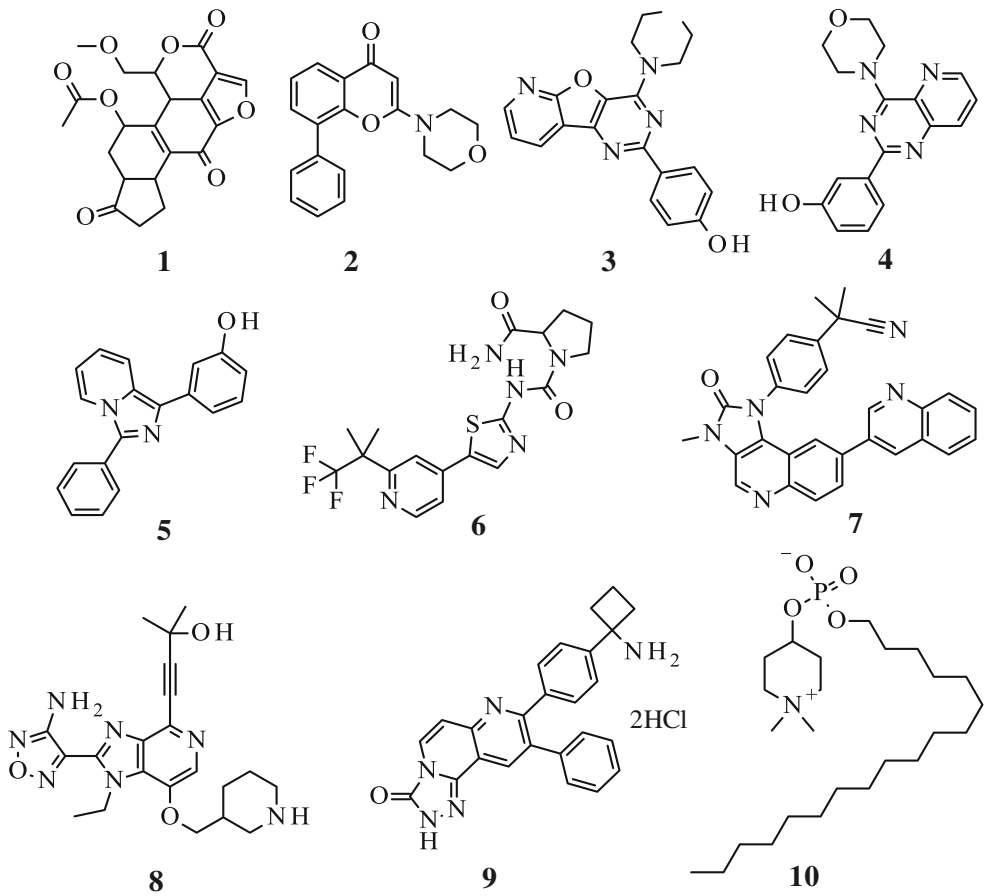

Fig. 1 Names and structures of inhibitors tested by capillary electrophoresis. *1* Wortmannin; CAS: 19545-26-7; 11-(acetyloxy)-1S, 6bR,7,8,9aS,10,11R, 11bR-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5-h]-2-benzopyran-3,6,9-trione. *2* LY294002; CAS: 154447-36-6; 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one. *3* PI-103; CAS: 371935-74-9; 3-[4-(4-morpholinyl) pyrido [3′,2′:4,5]furo[3,2-d]pyrimidin-2-yl]-phenol. *4* FB; 3-(4-morpholinopyrido[3,2-d]pyrimidin-2-yl)phenol. *5* FB181; 3-(3-phenylimidazo[1,5-a]pyridin-1-yl)phenol. *6* BYL719; CAS: 1217486-61-7;(2S)-1-*N*-[4-methyl-5-[2-(1,1,1-trifluoro-2-methylpropan-2-yl)pyridin-4-yl]-1,3-thiazol-2-yl]pyrrolidine-1,2-dicarboxamide. *7* BEZ235; CAS915019-65-7; 4-[2, 3-dihydro-3-methyl-2-oxo-8-(3-quinolinyl)-1H-imidazo[4,5-c]quinolin-1-yl]-α,α-dimethyl-benzeneacetonitrile. *8* GSK690693; CAS: 937174-76-0; 3-butyn-2-ol,4-[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-[(3S)-piperidinylmethoxy]-1H-imidazo[4,5-c] pyridin-4-yl]-2-methyl. *9* MK-2206; CAS: 1032350-13-2; 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2H-[1,2,4]triazolo[3,4-f][1,6] naphthyridin-3-one dihydrochloride. *10* Perifosine; CAS: 157716-52-4; 4-[[hydroxy(octadecyloxy)phosphinyl]oxy]-1,1-dimethyl-piperidinium, inner salt

Liquid handling was performed with micropipettes purchased from Fisher (Illkirch, France).

TR-FRET and luminescence technique

Human PI3Kα (specific activity 428 nmol/min/mg), PI3Kγ (167 nmol/min/mg), and PI3Kδ (509 nmol/min/mg) were supplied by Invitrogen. PI3Kβ (17.4 nmol/min/mg) was acquired from SignalChem (Richmond, Canada). 1,2-Dioctanoyl-*sn*-glycero-3-[phosphoinositol-4,5-biphosphate] and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). ATP was provided in the assay kits. Other buffer reagents such as dimethyl sulfoxide were purchased from Eurobio (Courtaboeuf, France) and Sigma-Aldrich. Liquid handling was performed with an electronic pipette Acura® electro 925 with a multichannel adaptor both purchased from Socorex (Lausanne, Switzerland). Vesicles of PIP_2 were first prepared by dissolving 500 μg of 1,2-dioctanoyl-*sn*-glycero-3-[phosphoinositol-4,5-biphosphate] in a suitable buffer (50 mM HEPES, 4 mM $MgCl_2$, 2 mM EGTA, and 0.1 % CHAPS) to obtain a final concentration of 1 mM. PS was then added to give a solution with an equimolar ratio between phosphatidylinositol 4,5-diphosphate (PIP_2) and PS. The mixture was then sonicated at 50/60 Hz (80 W/117 V) for an hour while keeping the bath temperature at 30±3 °C. A series of five cooling/thawing steps was then carried out with vigorous vortexing between each step. The PIP_2 was then stored at −80 °C. Adapta™, Transcreener™ and ADP-Glo™ assay kits exploited these vesicles as substrate for each enzymatic

reaction whereas HTRF PI3K™ and TR_{UE} FRET™ kits provided purified PIP_2. The equivalence of the prepared PIP_2 vesicles with the purified PIP_2 provided by these two kits was assessed. Other reactants used for kit inhibition assays were similar to those used for CE analyses.

Instrumentation and assay parameters

Capillary electrophoresis assays

The capillary electrophoresis system used was a 1600 Hewlett Packard^{3D}CE (Agilent, Waldbronn, Germany) equipped with a photodiode array detection system. Agilent software 3D-CE Chemstation (rev B.04.02) was used to pilot the CE system and for signal acquisition.

CE analyses were performed in fused-silica capillaries (66 cm total length, 57.5 cm effective length, 50 μm i.d.) purchased from Polymicro Technologies (Phoenix, AZ, USA). New capillaries were conditioned by 1.0 M NaOH for 30 min and water for 5 min. They were then coated by rinsing with cationic PDADMAC solution (0.2 % *w*/*v*) for 10 min from the anode side followed by 10 min from the cathode side [31]. After a waiting period of 5 min, the capillary was rinsed with water for 5 min and with background electrolyte (BGE) for 10 min. The coating was stabilized by applying an electric field of +30 kV for 10 min. The electroosmotic flow was anodic; it was measured by using DMSO as a neutral marker [32, 33]. The separation was conducted at −30 kV (reverse polarity) and at 30 °C. All rinse cycles were carried out at 4 bar. Between runs, the BGE in the separation vials was renewed and the capillary was flushed for 1 min with water, 2 min with PDADMAC, and 3 min with BGE.

All solutions were prepared with pure water, filtered through a 0.45-μm PVDF filter, and stored at 4 °C. For PI3K and mTOR assays, the incubation buffer was HEPES/NaOH/$MgCl_2$ (25 mM/8.84 mM/5 mM). Its pH was 7.2, and its ionic strength was about 60 mM. For Akt/PKBα (Akt1) assays, the incubation (reaction) buffer was HEPES/NaOH/$MgCl_2$ (25 mM/8.84 mM/10 mM); its pH was 7.2 and its ionic strength was about 75 mM. The BGE was tris/phosphate/$MgCl_2$ (117 mM/62.5 mM/5 mM); its pH was 7.2 and its ionic strength was 180 mM (Table S1, Electronic supplementary material). All buffers were prepared fresh each day. Their pH was measured with a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France).

Stock solutions of ATP (2 mM) and ADP (2 mM) were prepared in the incubation buffer and diluted to 50 μM. Inhibitor stock solutions were prepared by dissolving 1 mg in 1 mL of incubation buffer and DMSO (<4 % *v*/*v*). Aliquots of stock solutions of PI3 kinase isoforms (α, β, γ, and δ), Akt/PKBα, FRAP1/mTOR, PIP_2, crosstide, and mTOR substrate were diluted to 2, 9, 10, 2, 0.174, and 1.25 g L^{-1}, respectively. In all enzymatic assays, after incubation, the electric field (of −30 kV) was applied to separate the ADP formed during the enzymatic reaction from the other reactants. ADP was detected at 254 nm (bandwidth, 10 nm). No inhibitor plug was injected to determine the maximum activity of the enzyme; it was replaced by a plug of incubation buffer. Control blank assays were done without injecting the enzyme. ATP concentrations were set in all assays to be identical to those used in conventional methods (assay kits) for further comparison. For IC_{50} determination, enzyme and substrate concentration remained constant whereas at least 11 concentrations of inhibitors were used around the reported IC_{50} value in order to precisely determine IC_{50}. Assays were performed in triplicate (n=3). The dose–response curve for enzyme inhibition was carried out by plotting the enzyme activity (%) versus log [inhibitor] (Eq. 1). GraphPad Prism® (GraphPad Software, Inc., La Jolla, CA, USA) was used for curve fitting and thus for IC_{50} calculations using the following four parameter logistic equation:

$$\%\ A = \%\ A_{\min} + \frac{\%\ A_{\max} - \%\ A_{\min}}{1 + 10^{\log(IC_{50} - [I]) \times H}} \quad (1)$$

where $\%\ A$ is the enzyme activity (response), $\%\ A_{\min}$ and $\%\ A_{\max}$ are the minimal (for maximum inhibition) and the maximal residual enzyme activity, respectively, $[I]$ is the inhibitor concentration, and H is the Hill slope factor.

The enzyme activity (%) was defined as the ratio of the corrected peak area (CPA) of ADP formed in the presence and the absence of the inhibitor. The CPA was defined as the peak area divided by the migration time. The CPA of ADP was corrected by removing the CPA obtained for the blank assay.

The CE assay for studying PI3K (α, β, γ, and δ), Akt1, and mTOR kinases was based on using the capillary as a nanoreactor. TDLFP was used to in-capillary mix reactants and hence to trigger the enzymatic reaction. Plug interpenetration was simulated using the software reported by Krylov et al. [6, 34]. More details on the use of this approach for kinase analysis can be found in reference [12].

In the optimal conditions found in the present study, the injection sequence consisted in introducing the incubation buffer (25 mbar × 50 s; 2.2 % of the capillary effective length) followed by the injection at 50 mbar for 5 s (7.3 nL; 0.5 % of the capillary effective length) of PI3K (2 mg L^{-1}), substrate PIP_2 (2 g L^{-1}), incubation buffer (or inhibitor in inhibition assays), ATP (50 μM; 0.027 g L^{-1}), and then PI3K (2 mg L^{-1}) a second time. The incubation buffer was injected (50 mbar × 30 s; 1.6 % of the capillary effective length) at the end of the injection sequence, and reactants were then incubated for 10 min.

Akt1 assays were conducted by injecting the incubation buffer (25 mbar × 50 s) followed by the injection at 50 mbar for 5 s of Akt1 (9 mg L^{-1}), substrate crosstide (0.174 g L^{-1}),

incubation buffer (or inhibitor in inhibition assays), ATP (50 μM), and incubation buffer (or inhibitor in inhibition assays) a second time. Then, the incubation buffer (50 mbar×30 s) was injected at the end of the injection sequence, and reactants were incubated for 10 min.

mTOR assays were performed by injecting the incubation buffer (25 mbar×50 s) followed by the injection at 50 mbar for 5 s of FRAP1/mTOR (10 mg L^{-1}), mTOR substrate (1.25 g L^{-1}), incubation buffer (or inhibitor in inhibition assays), ATP (50 μM), and then FRAP1/mTOR (10 mg L^{-1}) a second time. The incubation buffer was injected (50 mbar× 30 s) at the end of the injection sequence and reactants were also incubated for 10 min.

In order to assess day-to-day reproducibility, IC_{50} determination was done independently at intervals of several days. It was evaluated by calculating the Z' factor (Eq. 2) [35]:

$$Z' = 1 - \left[\frac{3 \times (\sigma_{c^+} + \sigma_{c^-})}{\mu_{c^+} - \mu_{c^-}}\right] \quad (2)$$

In which μ is the mean, and σ is the standard deviation of the positive c^+ or the negative control c^-. c^- corresponds to 100 % inhibition (either 5 μM of PI-103 or no ATP for instance) and c^+ corresponds to 0 % inhibition (no inhibitor in the enzymatic reaction).

A Z' equal to 1 is considered ideal, between 0.5 and 1 indicates a robust assay kit, whereas under 0.5 the assay will be evaluated as unreliable and not reproducible.

The S/B was calculated for the lowest assay response in order to have the most significant data concerning the sensitivity of the test.

TR-FRET and luminescence assays

All TR-FRET and luminescence assays were performed in low volume white 384-well plates acquired from Corning (ref: 3674). Time-resolved fluorescence was measured using a Victor V plate reader (Perkin Elmer) while luminescence was quantified using a Mithras LB 940 plate reader (Berthold). Excitation (Ex1) and emission (Em1, Em2) wavelengths are summarized in Table S2 (Electronic supplementary material). The measurement height for the reading was 8 mm from the bottom of the plate. The counting delay was 100 μs, 200 μs for the counting window and 1,000 μs for each counting cycle. The integration time for luminescence was set at 1 s per well. All inhibitors were dissolved to the desired concentration and then diluted in a twofold serial dilution in DMSO. Inhibitor solutions were then diluted in the incubation (reaction) buffer in order to have a final DMSO concentration of 2 % in each well. The composition of the incubation and detection buffers is described in Table S1 (Electronic supplementary material). Each optimization curve and IC_{50} determination was done with 16 points in triplicate. Day-to-day reproducibility of the IC_{50} values was also assessed as for the CE assays. All kinase reactions were performed in a final volume of 10 μL except for ADP-Glo™ and HTRF PI3K™ where reaction volumes of 5 and 20 μL were respectively used. All kinases were diluted in the related incubation buffer to obtain a solution that was four times more concentrated than the value obtained in the optimization. The ATP solution was prepared from a concentrated stock solution at 10 mM. It was then diluted in the related reaction buffer with PIP_2 to obtain a solution twice as concentrated as in the reaction, except for HTRF PI3K™ where it was four times as concentrated. All ATP and substrate levels were below the K_m value (30 and 50 μM, respectively). For assay kits where the tracer is ADP-competitive, the levels of PIP_2 were equal to the K_m value so as to avoid any influence on the outcome. For the assay monitoring the levels of PIP3, the level of substrate was lower to have similar competition. The principle of the kits based on TR-FRET is presented in Fig. S1.b (Electronic supplementary material).

As for CE assays, the reproducibility of each kit was assessed by calculating the Z' factor (Eq. 2), and its sensitivity was evaluated by determining the S/B ratio.

TR-FRET Adapta™

The Adapta™ assay kit (Invitrogen, PV5099, Life Technologies) uses a tracer in the form of ADP linked to a chromophore acceptor and an antibody anti-ADP linked to a chromophore donor. Depending on the efficiency of the inhibitor, the level of ADP (which competes with the tracer) will vary, thereby directly affecting the binding of the tracer to the antibody. The TR-FRET signal will evolve accordingly [36]. The working concentrations were 10 μM for ATP (which is below the K_m) [37] and 50 μM for PIP_2 (equal to the K_m) [38]. The experimental process is the following: the inhibitor solution (2.5 μL) was injected in a plate followed by the addition of 2.5 μL of PI3K (α, γ, or δ) and ATP/PIP_2 (5 μL) solutions. After incubating for an hour at room temperature with no agitation and with the plate sealed, the kinase reaction was stopped by adding the detection solution to yield final concentrations of 10 nM for the tracer, 2 nM for the antibody, and 30 mM of EDTA (5 μL). All of these compounds were diluted in a detection buffer (PV3574, Invitrogen), and the plate was sealed and incubated for the optimal time of 30 min at room temperature without shaking before the reading step.

TR-FRET Transcreener™ ADP

As for the Adapta™ kit, the principle of Transcreener™ (Bell Brook Labs, 3011-1K, Madison, WI, USA) is based on evaluation of the ADP levels [29]. The protocol is the same; the concentrations were 10 μM for ATP, 50 μM for PIP_2, 13.6 nM

for tracer, and 4 nM for antibody. The incubation time for the detection is 1 h.

HTRF Transcreener™

The protocol of HTRF Transcreener™ (Cisbio Bioassays, 62ADPPEB, Bagnols sur Cèze, France) is the same as for the previous two kits. The concentrations were set at 10 μM for ATP and 50 μM for PIP_2. The incubation time for the detection is 1 h.

TR-FRET PI3-Kinase HTRF™

The PI3K HTRF™ (Millipore, 33-017, Billerica) kit consists in monitoring the level of PIP3. It exploits a PIP3 detector in the form of a chromophore donor linked, by an antibody, to a PH domain which binds to PIP3 and a tracer that is PIP3 linked to a chromophore acceptor. Depending on the efficiency of the inhibitor, the level of PIP3 will vary, thereby competing with the tracer in binding to the PH domain. The TR-FRET signal will evolve accordingly; a high TR-FRET signal indicates a low PI3K activity. The working concentrations were 10 μM for ATP and 10 μM for PIP_2. The experimental process is the following: the inhibitor solution (0.5 μL) was injected in a plate well with the PI3K/PIP_2 (14.5 μL) solution followed by the injection of the ATP solution (5 μL). After incubating for an optimal time of 30 min at room temperature with no agitation and with the plate sealed, the kinase reaction was stopped by adding the Stop Solution. The detection mixture (5 μL) was added, and the plate was sealed and incubated for an optimal period of 6 h at room temperature.

ADP-Glo™

The ADP-Glo™ kinase assay kit (Promega, V9101, Charbonnières les bains, France) is based on bioluminescence by using an ATP detector. After the kinase reaction has occurred, a reagent is added to stop the reaction and deplete the ATP that was not consumed by the reaction. Another reagent is then added to convert the ADP generated into fresh ATP. Then, using a luciferase/luciferin reaction, the levels of ATP can be measured by luminescence. Depending on the efficiency of the inhibitor, the level of ADP and thus ATP will vary, and the signal will evolve accordingly. The working concentrations were 10 μM for ATP and 50 μM for PIP_2. The inhibitor and ATP were mixed together (as they are competing in binding to the kinase) as well as PIP_2 with PI3K (α, γ, or δ). The experimental process is the following: both ATP/inhibitor (2.5 μL) and PIP_2/PI3K (2.5 μL) solutions were injected in each well. After incubating during the optimal time of 2 h at room temperature with no agitation and with the plate sealed, the kinase reaction was stopped by adding the ADP-Glo™ Reagent solution (5 μL), and the plate was sealed and incubated for 40 min at room temperature with shaking in order to deplete the unconsumed ATP. The kinase reaction was then stopped by adding the kinase detection solution (10 μL). The plate was then sealed and incubated for 30 min at room temperature with shaking before reading.

All experiments, including the optimization steps and the inhibition curves were performed in triplicate. As for CE assays, all data were plotted and analyzed using GraphPad Prism® software (Eq. 1). The response of the assay to ADP formation is not linear with the Adapta™ assay kit, whereas the response of the other tests takes this aspect into account and is thus linear. Therefore, with Adapta™, the amount of inhibitor that causes a change of 50 % in TR-FRET intensity can be considered as the relative IC_{50}. In order to calculate the absolute IC_{50}, a calibration method was setup. A 10 μM solution of ADP was diluted in a 1.5-fold serial dilution in solutions of 10 μM ATP. The top solution containing only ADP (maximum concentration) will correspond to 100 % conversion of ATP into ADP, and the bottom solution containing traces of ADP (minimum concentration) will represent a 0.2 % conversion of ATP into ADP. Each solution was added in a well along with the antibody (2 nM) and the tracer (10 nM). The ADP and tracer then compete for binding to the antibody, and the TR-FRET response evolves accordingly. An ATP–ADP titration curve is thus obtained by plotting the TR-FRET emission ratio versus the conversion percentage (ATPADP). The data can thus be fitted to a three-parameter hyperbolic model in GraphPad Prism®, with three curve–fit variables called A, B, and C. Once these variables determined by the software (A=0.096, B=2.759, and C=0.007) had been obtained, the emission ratio values from the inhibitor assay were converted into % Conversion using Eq. 3:

$$\%\ \text{Conversion} = B\left(\frac{C + A - \text{Ratio}}{\text{Ratio} - C}\right) \tag{3}$$

The % Conversion value was then plotted versus the concentration of inhibitor, and the data were fitted to a sigmoidal dose–response curve with a variable slope. The 50 % TR-FRET response change obtained from this curve can be considered as the absolute IC_{50}.

Results and discussion

Optimization steps for the CE-based kinase assay on PI3Kα isoform

The in-capillary enzymatic assays on PI3Kα were conducted based on the capillary electrophoresis method that we previously developed for studying human cell cycle protein kinase inhibition [12]. A simple and rapid optimization was

conducted to adapt this CE assay to the analysis of the α isoform of the PI3K lipid kinase. In-capillary CE has never been used for studying signaling pathway kinases. The ADP was quantified to monitor the enzymatic activity, and special attention was paid to quenching any reactant adsorption to the capillary wall. In fact, adsorption was observed in the present study since poor repeatabilities were obtained; RSD $CPA_{(ADP)}$ was about 15 % (n=6) for enzymatic assays conducted in bare silica capillaries. In our previous work, the inner capillary wall was coated with a cationic polymer, polybrene. This polymer was used to obtain a dynamic coating which is not stable unless the coating agent is introduced in the BGE during analyses. In this work, the highly charged polymer PDADMAC was chosen instead of polybrene. It has been shown [33, 39] that when new capillaries were simply rinsed with this polyelectrolyte, a stable static cationic coating was obtained even in the absence of PDADMAC in the BGE. The advantage of this approach is that it limits the risk of denaturing the enzyme in the capillary. The PDADMAC coating also reverses the electroosmotic flow, thereby accelerating the migration of the anionic molecules [32], especially ADP that was detected at about 4.5 min (Fig. 2). The ADP peak symmetry (0.9±0.1) and efficiency (~200,000 theoretical plates) were very good. Resolution between ADP and ATP was about 6.5. Very good repeatabilities were obtained with RSD $t_{m(ADP)}$ below 3.5 % and RSD $CPA_{(ADP)}$ below 4.2 % (n=6). The interday RSDs on migration times and on peak areas were below 5 %, showing that the CE analyses were reproducible.

When conducting an in-capillary PI3Kα reaction with TDLFP, the most important parameter to consider is the diffusion coefficient D ($cm^2\ s^{-1}$) of the reactants. D was obtained from the literature (D_{ATP} ~4.5×10^{-6} [40]) or estimated using the Stokes–Einstein equation [41]; $D_{PI3K\alpha}$ ~6×10^{-8}, D_{PIP2} ~1.2×10^{-6}, and $D_{inhibitor}$ ~5×10^{-6} $cm^2\ s^{-1}$. The York number (Yo), a dimensionless parameter that can predict the plug shape during injection, was then calculated for each reactant and found to be relatively low, approximately between 0.02 for the reactant with the lowest D and 3.6 for the reactant with the highest D. Indeed, to obtain good reactant mixing by TDLFP, Yo must be as low as possible [42]. To achieve this, injecting the enzymatic reactants at 50 mbar pressure during 5 s (or less) was considered to be adequate to obtain parabolic plug profiles. Relatively short transverse diffusion times were obtained, ranging from 1.4 s (for ATP or inhibitor) to 110 s (for PI3K). This proves that, in these conditions, TDLFP is very interesting for conducting fast in-capillary reactant mixing for an inhibition study of PI3Kα. Moreover, a plug of the incubation buffer must be introduced at the beginning of the injection sequence to isolate the reaction mixture from the BGE. The best results were obtained in this study when injection was conducted at 25 mbar pressure for 50 s. Another plug of incubation buffer must be injected at the end of the injection sequence; however, this induces an important dilution of the reactants inside the capillary [43]. In a previous study by our group, this dilution factor was successfully corrected [13]. In this study, the best results were obtained by injecting the incubation buffer at 50 mbar for 30 s at the end of the injection sequence.

Moreover, assays showed that the double injection of the enzyme (PI3Kα) resulted in a better reactant mixing. Indeed, reactant double injection is expected to improve mixing for kinase inhibition assays [12]. This can be explained by the wide range of diffusion coefficients of the reactants, going from about 5.10^{-6} $cm^2\ s^{-1}$ for ATP and potential inhibitor to about 2.10^{-8} $cm^2\ s^{-1}$ for PI3Kα. Hence, five plugs were successively injected into the capillary. Once mixing by TDLFP occurred in the capillary, the reaction mixture was allowed to incubate in order to accumulate ADP. Incubation times between 0 and 30 min were tested, and it was found that 10 min of incubation was enough; about 5 μM of ADP were produced in the absence of any inhibitor (lower limit of quantification of ADP about 0.2 μM). It is important to note that all these injection steps are fully automated. Moreover, since five plugs were injected, the concentration of ATP was 50 μM so that after mixing all the injected plugs, the final ATP concentration (10 μM) would be equal to the one used in the kits.

Evaluation of the CE-based kinase assay method on PI3Kα

The CE-based kinase assay described previously was compared to commercial TR-FRET and bioluminescence kits to measure the inhibition of PI3Kα activity by different molecules. In a first approach, the inhibition of PI3Kα by three commercial inhibitors, wortmannin, LY294002 and PI-103 (compounds 1, 2 and 3 in Fig. 1) was conducted. IC_{50} values, Z', S/B, and r^2 were determined using each method. The inhibitors were chosen because wortmannin (compound 1) is a natural steroid molecule that is a very efficient inhibitor but lacks selectivity towards PI3K isoforms. It is a first-generation inhibitor like the synthesized molecule LY294002 (compound 2). Wortmannin is an irreversible ATP inhibitor that is very toxic. PI-103 (compound 3) is however a second-generation inhibitor which is less toxic and more specific than the first generation.

Figure 2a shows the electropherograms obtained during in-capillary inhibition of PI3Kα by different PI-103 (compound 3) concentrations. As can be seen, the ADP peak is inversely proportional to the PI-103 concentration. Even at the highest concentration of the studied inhibitors, the ADP obtained was still above the LOQ which is important for precise quantification. Figure 2d shows dose–response curves obtained during the in-capillary inhibition of PI3Kα by wortmannin, LY294002, and PI-103 (compounds 1–3). Results in terms of IC_{50}, Z', and r^2 values are summarized in Table 1. Good fitting ($r^2 \geq 0.995$) and high Z' values (Z' ~0.9) were obtained showing the robustness and the reliability of the technique.

Compounds 1–3 were also tested on PI3Kα using each commercial assay kit as described by the manufacturer or in the conditions we previously optimized. The IC_{50}, Z', and r^2

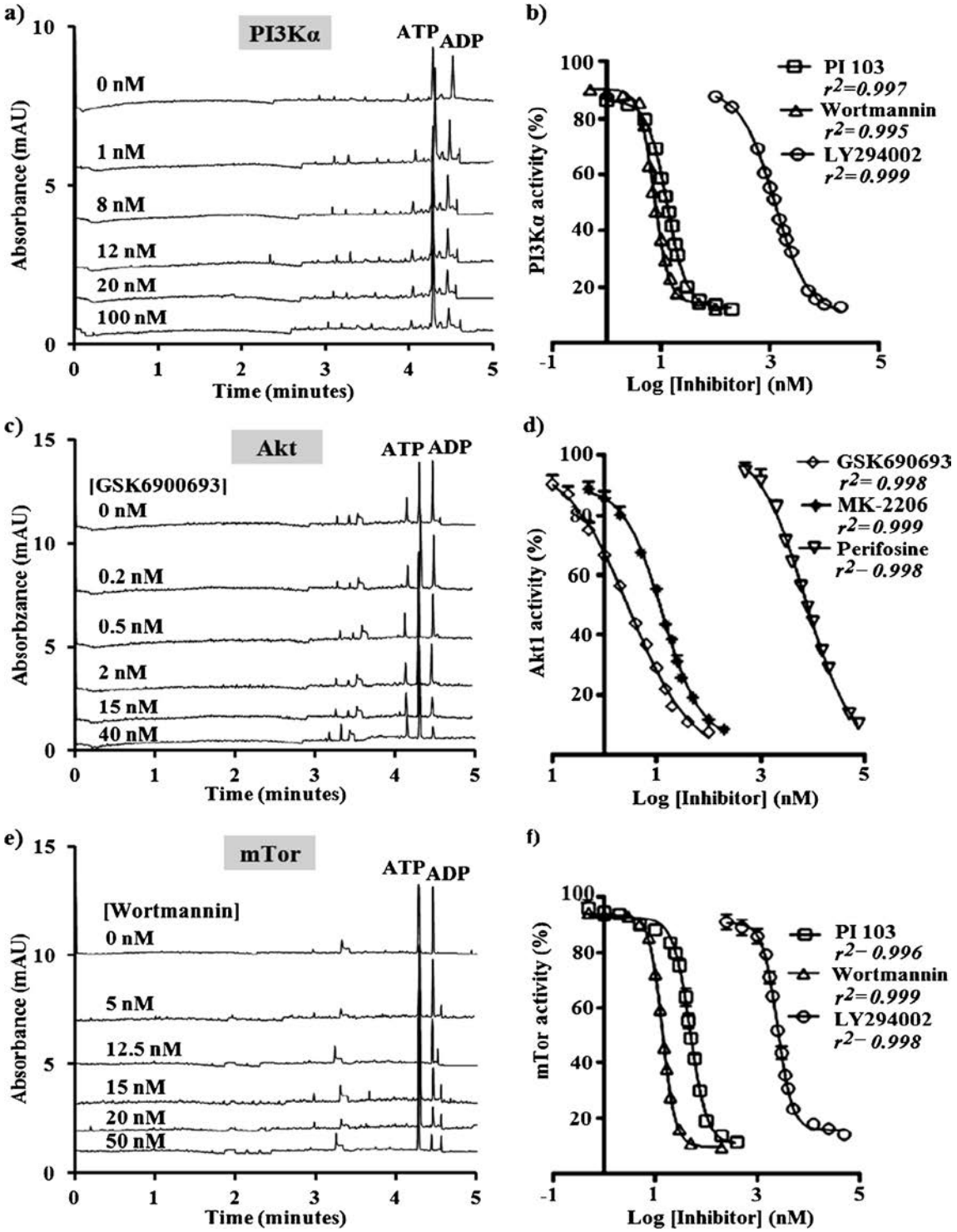

Fig. 2 Electropherograms obtained for in-capillary inhibition of (**a**) PI3Kα by PI-103, (**b**) Akt1 by GSK690693, and (**c**) mTOR by wortmannin. Dose–response curves obtained for inhibition of (**d**) PI3Kα by PI-103, wortmannin, and LY294002; (**e**) Akt1 by GSK690693, MK-2206, and perifosine; and **f** mTOR by PI-103, wortmannin, and LY294002

values obtained are also summarized in Table 1. All the assays yielded IC_{50} values comparable to those reported in the literature [16, 20, 23, 24, 29]. However, the results obtained with the HTRF PI3K^{TM} kit cannot be considered trustworthy since Z' was in some cases below 0.4. In fact, with this kit, even in the presence of a plate seal, 6 h of incubation cause the evaporation of the solution, and the assay signal was not stable enough at room temperature resulting in high standard deviations. Adapta™, ADP-Glo™, and both Transcreener kits seem to be reliable because the parameters considered (Z'

Table 1 Results obtained for the inhibition of PI3Kα by three reference inhibitors

Assay	Results	Wortmannin	LY294002	PI-103
Published IC_{50} (nM) on PI3Kα [20, 23, 24]		4–5	1,000	5–9
Capillary electrophoresis (CE)	IC_{50} (nM)	7.5±0.5	1,224.0±61.0	10.2±1.4
	Z'	0.917	0.894	0.853
	r^2	0.995	0.997	0.996
Adapta™	IC_{50} (nM)	5±1	759±111	6±1
	Z'	0.598	0.718	0.768
	r^2	0.989	0.988	0.992
Transcreener™	IC_{50} (nM)	2±0	936±101	8±4
	Z'	0.572	0.581	0.623
	r^2	0.962	0.978	0.989
HTRF Transcreener™	IC_{50} (nM)	3±1	1,173±254	22±4
	Z'	0.646	0.609	0.573
	r^2	0.986	0.99	0.979
HTRF PI3K™	IC_{50} (nM)	6±1	*n.d.*	18±3
	Z'	0.163	*n.d.*	0.338
	r^2	0.939	*n.d.*	0.920
ADP-Glo™	IC_{50} (nM)	7±1	1,386±129	10±1
	Z'	0.617	0.867	0.801
	r^2	0.988	0.997	0.989

and r^2) are good. It can be seen that the best Z' values were obtained with the Adapta™ and the ADP-Glo™ kits; $Z' \geq 0.6$ and r^2 ~0.99. The ATP–ADP calibration of the Adapta™ assay probably enhanced linearity, thus reducing standard deviations. The ADP-Glo™ kit based on luminescence ensured a more stable signal (better standard deviations and amplitude) than the other kits. This is probably due to the fact that there is no competition between the tracer and ADP/PIP3. It is interesting to note that for kit assays in high-throughput screening, pipette robots would probably provide better Z' values than those presented in the manuscript. Moreover, the Z' and r^2 parameters obtained show that the CE-based kinase assay developed here is highly efficient, reliable, and reproducible. Furthermore, it appears to be economic and requires a rapid (10 min) incubation time and simple optimization.

Evaluation of the CE-based kinase assay method on the other isoforms of PI3K (β, γ, and δ)

As for PI3Kα, compounds 1 to 3 were tested on the PI3K isoforms β, γ, and δ with the CE-based kinase assay. CE has never been used to measure the IC_{50} of inhibitors of these PI3K isoforms. The two commercial kits exhibiting the best robustness and reliability (Adapta™ and ADP-Glo™) were also used for comparison. Note that exactly the same CE assay was used for the different isoforms of PI3K. No further optimization was needed since the method is based on monitoring ADP production which is common for all ATP-binding kinases. Moreover, identical in-capillary TDLFP mixing is conducted because the diffusion coefficients for the (β, γ, and δ) PI3K isoforms are close to that of the α isoform. Results in terms of IC_{50}, Z', S/B, and r^2 values are summarized in Table 2.

IC_{50} values were successfully determined by CE for all PI3K isoforms. Results with good variability were obtained ($r^2 \geq 0.98$), as with Adapta™ and ADP-Glo™. Very good reproducibility ($Z' \geq 0.9$) and sensitivity (S/B≥10.0) were found with the CE-based enzymatic assay, showing its efficiency and robustness. CE has been found to be an interesting novel technique for studying the inhibition of the four isoforms of PI3K (α, β, γ, and δ). For this reason, the inhibition of PI3K isoforms by two compounds synthesized by our group (compounds 4 and 5; Fig. 1) was evaluated using this method. Two powerful commercial inhibitors, BYL719 and BEZ235 (compounds 6 and 7) were also tested. They belong to the third generation of kinase inhibitors, and are thus less toxic and more selective than inhibitors of the first and second generation. BYL719 is described as an investigational selective inhibitor of PI3Kα whereas BEZ235 is a dual inhibitor of PI3K and mTOR. These two compounds are currently undergoing phase I/II clinical trials for solid tumors [44–46]. Table 3 shows that IC_{50} values determined by CE were comparable to the values determined by kit assays and reported in the literature [20, 23–30]. The tested compounds have a wide range of IC_{50} values toward PI3K isoforms, going from a few nanomolars to a few micromolars. Some of them such as compounds 4 and 5 were not specific toward a

Table 2 Comparison of results obtained by different techniques for PI3K isoforms inhibition by PI-103

Assay	PI3K	[PI3K] (ng/mL)	Specific activity (nmol/min/mg)	[ATP] (μM)	[PIP_2] (μM)	Published IC_{50} (nM) for PI-103	Obtained IC_{50} (nM) for PI-103	Z'	S/B	r^2
CE	PI3Kα	2,000	565	10	14	*n.a.*	10.2±1.3	0.894	10.3	0.997
	PI3Kβ	2,000	246	10	14	*n.a.*	105.6±8.0	0.917	10.5	0.997
	PI3Kγ	2,000	23	10	14	*n.a.*	29.2±2.1	0.934	10.0	0.998
	PI3Kδ	2,000	348	10	14	*n.a.*	4.4±0.2	0.946	10.8	0.999
Adapta ™	PI3Kα	494.5	42.3	10	50	5.9[a]	6.4±1.0	0.768	4.3	0.992
	PI3Kβ	–	–	–	–	*n.a.*	*n.d.*	–	–	–
	PI3Kγ	1,347	44.5	10	50	28[a]	47.1±7.1	0.713	4.1	0.995
	PI3Kδ	412	83.8	10	50	3.1[a]	2.3±0.3	0.744	7.8	0.988
ADP-Glo™	PI3Kα	1,100	95	10	50	8[b]	10.0±1.1	0.801	5.8	0.988
	PI3Kβ	–	–	–	–	88[b]	*n.d.*	–	–	–
	PI3Kγ	1,780	59.4	10	50	150[b]	45.5±7.7	0.706	5.1	0.981
	PI3Kδ	948	193	10	50	48[b]	15.5±0.7	0.788	4.7	0.996

n.a. Not available. *n.d.* not determined

[a] Reference values obtained exclusively with Adapta™ using TR-FRET technique published by Invitrogen [36]

[b] Reference values obtained by autoradiography technique [23]

particular isoform of PI3K; they are called "pan-specific" [47, 48]. Some others had a low inhibitory effect on PI3K such as the home-made compound 5 as well as LY294002 (compound 2); however, the latter has some affinity

Table 3 IC_{50} values obtained during CE inhibition assays realized on PI3K isoforms, Akt1, and mTOR

	IC_{50} (nM)					
Compound	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ	Akt1	mTOR
Wortmannin (1)	7.5±0.5	9.0±1.2	48.3±3.6	15.8±1.2	*n.d.*	12.7±0.2
	4–5 [20, 23, 24]	*9–19* [29]	*2–50* [20, 23, 24]	*9* [26]	–	*4–15* [20, 21]
LY294002 (2)	1,224.0±61.0	407.5±40.9	2,846.0±234.0	1,197.0±85.0	*n.d.*	2,223.0±74.0
	1,000 [20, 23, 24]	*300–2,900* [20, 23, 24]	*2,800–5,600* [20, 23, 24]	*570–980* [25, 28]	–	*1,000* [20, 21]
PI-103 (3)	10.2±1.4	105.6±8.0	29.2±2.1	4.5±0.2	*n.d.*	41.8±2.0
	5–9 [20, 23, 24]	*13–88* [20, 23, 24]	*28–47* [20, 23, 24]	*3–48* [20, 23, 24]	–	*23–27* [20, 21]
4	27.2±1.0	7.7±0.4	33.8±2.4	4.2±0.2	*n.d.*	*n.d.*
	19 [20]	*n.a.*	*31* [20]	*3* [20]	–	–
5	2,804.0±153.0	2,077.0±93.0	3,145.0±152.0	2,837±146	*n.d.*	*n.d.*
	n.a.	*n.a.*	*n.a.*	*n.a.*	–	–
BYL719 (6)	21.0±2.2	64.2±6.9	69.5±6.2	882.6±52.1	*n.d.*	*n.d.*
	n.a.	*n.a.*	*n.a.*	*n.a.*	–	–
BEZ235 (7)	6.8±0.4	91.5±6.5	4.6±0.3	14.1±0.7	*n.d.*	*n.d.*
	4 [27]	*75* [27]	*5* [27]	*7–8* [27]	–	–
GSK690693 (8)	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.7±0.2	*n.d.*
	–	–	–	–	*2* [19]	–
MK-2206 (9)	*n.d.*	*n.d.*	*n.d.*	*n.d.*	11.5±1.2	*n.d.*
	–	–	–	–	*8* [22]	–
Perifosine (10)	*n.d.*	*n.d.*	*n.d.*	*n.d.*	6,127.0±588.0	*n.d.*
	–	–	–	–	*4,700* [18]	–

Compound structures, Fig. 1. IC_{50} values obtained by conventional methods are presented in italic.

n.a.: not available in literature. *n.d.*: not determined by CE

toward PI3Kβ. BEZ235 (compound 6) is very active on PI3K α, γ, and δ. BYL719 (compound 7) was found, as reported in the literature, to have higher affinity toward PI3Kα (IC_{50}=21.0±2.2 nM); however, it was also found to be very active on PI3Kβ and δ. Based on these results, compound 4—which was synthesized by our group—as well as BYL719 (compound 7) were found to be very promising anticancer agents since they have high affinity toward PI3K.

The CE-based PI3K assay provided reliable IC_{50} values ($Z'\geq0.9$, S/B≥9, and $r^2\geq0.995$). The economy and the simplicity of this technique make it a promising technique for evaluating kinase inhibitors. In the following, the same CE approach was employed to conduct inhibition assays on the other two protein kinases of the pathway: Akt1 and mTOR. CE has never been used to measure IC_{50} values of Akt or of mTor inhibitors.

Evaluation of the CE-based kinase assay method on Akt1 and mTOR

Three inhibitors were tested on Akt1 (compounds 8, 9, and 10) and three on mTOR (compounds 1, 2, and 3) using the CE-based kinase assay method. MK-2206 (9) is in a phase II trial study, and perifosine (10) is an anticancer drug. Assays were conducted as previously described in the "Experimental section". The electropherograms obtained for in-capillary inhibition of Akt1 by GSK690693 and of mTOR by wortmannin are shown in Fig. 2b, c, respectively. Figure 2e, f shows the dose–response curves of the inhibition of Akt1 and of mTOR by compounds 8–10 and 1–3, respectively. Excellent values were obtained for Z' and r^2 ($Z'\geq0.9$ and $r^2\geq0.996$), showing the robustness of the CE enzymatic assay on these kinases. The IC_{50} values obtained are summarized in Table 3. As can be seen, perifosine showed a small inhibitory effect (IC_{50}=6,127 nM) toward Akt1 contrary to GSK690693 (IC_{50}=2.73 nM) and MK-2206 (IC_{50}=11.45 nM). These values and conclusions were consistent with those obtained with conventional methods in the literature [18–22]. Compounds 1–3 have almost similar affinity toward mTOR and PI3Kα. This is not surprising in view of the homology between the structure of the kinase domain of PI3K and mTOR. Such inhibitors are called "dual PI3K/mTOR" inhibitors [4, 5].

Conclusion

Human kinases are high-impact pharmacological targets for drug testing. Good commercial kits, based on TR-FRET and bioluminescence, already exist for assaying the inhibition of kinases and appear to be reliable and reproducible. CE is becoming very interesting in this domain. In this work, a novel simple in-capillary assay was developed to assess the inhibition of kinases of the signaling pathway PI3K/Akt/mTOR. This assay takes advantage, for the first time, of the rapid in-capillary reactant mixing by TDLFP and of ADP quantification by simple UV detection. The CE assay was easily optimized for each kinase/substrate couple since it does not require any fluorescent or specifically modified substrates, antibodies, or radiolabeled ATP. Only a few tens of nanoliters of reactants were required to measure an IC_{50} value. Good Z' and r^2 ($Z'\geq0.9$ and $r^2\geq0.996$) were obtained, demonstrating the robustness of the CE-based enzymatic assay proposed here.

This work proves that TDLFP is a generic and simple method for in-capillary mixing of various reactants; lipids, peptides, proteins, and nucleotides. CE was shown to be a promising technique for conducting lipid as well as protein kinase assays economically. However, this approach does not provide the same number of assays a day as FRET and luminescence assays which can be conducted in 384-well plates. Using an automated multicapillary electrophoresis apparatus would thus be very interesting.

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