Analytical Biochemistry 473 (2015) 46–52

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Residence time and kinetic efficiency analysis of extracellular signal-regulated kinase 2 inhibitors

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ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 11 December 2014
Accepted 12 December 2014
Available online 20 December 2014

Keywords:
Structure–kinetic relationship
Kinetic efficiency
ERK2
Time-dependent inhibition
Off-rate
Residence time

ABSTRACT

The RAS/RAF/MEK/ERK signal transduction cascade plays an important role in the regulation of critical cellular processes such as cell proliferation, migration, and differentiation. The up-regulation of this pathway can negatively affect cell homeostasis and is responsible for the development of various forms of cancer and inflammation processes. Therefore, there is a strong interest in pursuing drug programs targeting some of the enzymes involved in this pathway. In addition to the determination of K_i, K_d, IC_{50}, and/or EC_{50}, a more thorough kinetic analysis can provide useful information for the selection of the best lead series during the early stage of the drug discovery process. This study describes a medium-throughput fluorescent probe displacement assay for the rapid determination of the k_{off} constant, residence time, and kinetic efficiency for ERK (extracellular signal-regulated kinase) inhibitors. Using this method, we have identified several inhibitors that we have subjected to further kinetic analysis by comparing k_{off} constants determined for these time-dependent inhibitors using either the active or inactive form of ERK2.

The mitogen-activated protein kinase (MAPK)[1] cascades play a critical role in cell signal transduction that leads to cell proliferation, survival, and differentiation. In mammals, there are several well-characterized and interrelated MAPK cascades that can be succinctly described as follows: MAP kinase kinase kinase (MAPKKK)–MAP kinase kinase (MAPKK)–MAP kinase (MAPK). Each enzyme of these pathways is activated in a sequential and coordinated fashion to the final activation of either extracellular signal-regulated kinase (ERK1/2), c-Jun amino-terminal kinase (JNK1/2/3), p38 kinase (p38α/β/γ/δ), or ERK5 kinase (see Ref. [1] for a review). The RAS/RAF/MEK/ERK signal transduction cascade has been widely investigated for its role in cancer, diabetes, inflammation, and cardiac and vascular diseases [2–5]. This cascade is stimulated by the binding of growth factors to the extracellular domain of receptor tyrosine kinases (e.g., EGFR, VEGFR). The resulting activated Tyr kinase will then initiate the recruitment of a large protein complex consisting of RAS:GTP, Grb2, SOS, and RAF, a Ser/Thr kinase that phosphorylates and activates MEK, followed by ERK1 and -2 downstream. Once activated, ERK kinases stimulate a vast array of genes that modulate numerous cellular responses such as cell growth, cell survival, motility, division, apoptosis, autophagy, morphology, and the epithelial–mesenchymal transition mechanism [2].

A large number of small molecule inhibitors targeting several enzymes of this pathway are now in various stages of the drug discovery and clinical development process. Table 1 (see also Refs. [4–7]) provides a short overview of the RAS, RAF, MEK, and ERK inhibitor landscape and their therapeutic areas. Recent advances in biochemical and biophysical screening technologies have provided many scientific labs with the necessary tools to explore another kind of relationship between a compound's intrinsic kinetic constants (i.e., k_{on} and k_{off}) and its physicochemical properties [8–10]. For the past 5 years, the new concept of structure–kinetic relationship (SKR) has emerged in many scientific publications and is now being increasingly used to investigate the impact of k_{off}, k_{on}, residence time, and kinetic efficiency (KE) on the in vivo efficacy of selected compounds [11].

In this study, we highlight a simple medium-throughput method for the determination of k_{off} constants. This method

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[1] *Abbreviations used:* MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RAS, rat sarcoma; RAF, rapidly accelerated fibrosarcoma kinase; MEK, mitogen-activated protein kinase kinase or MAP2K; EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; GTP, guanosine triphosphate; Grb2, growth factor receptor-bound protein 2; SOS, son of sevenless protein; SKR, structure–kinetic relationship; KE, kinetic efficiency; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TR–FRET, time-resolved fluorescence resonance energy transfer; NHS–PEG4, *N*-hydroxysuccinimide ester polyethylene glycol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SA–Eu, streptavidin-labeled europium; E–I, enzyme–inhibitor; ATP, adenosine triphosphate; SPR, surface plasmon resonance.

http://dx.doi.org/10.1016/j.ab.2014.12.008
0003-2697/

Table 1
Kinase inhibitors in various preclinical and clinical stages targeting the RAS/RAF/MEK/ERK signaling cascade.

	Target	Indication/Disease	Company	Status
Vemurafenib	BRAF (V600E)	Cancer (melanoma, brain, thyroid, myeloma)	Roche/ Daiichi Sankyo	FDA approved (melanoma) Phase II (brain, thyroid, myeloma)
Dabrafenib	BRAF (V600E)	Cancer (melanoma, brain, NSCLC, head and neck, colorectal)	GSK	FDA approved (melanoma) Phase II (brain, NSCLC, head and neck, colorectal)
Encorafenib	BRAF (V600E)	Cancer (melanoma, colorectal, thyroid)	Novartis	Phase III (melanoma) Phase II (colorectal, thyroid)
Trametinib	BRAF (V600E & V600K)	Cancer ((melanoma, leukemia, NSCLC, pancreatic, myeloma, head and neck, colorectal, breast, brain, biliary, endometrial)	GSK	FDA approved (melanoma) Phase II (leukemia, NSCLC, pancreatic, myeloma, head and neck, colorectal, breast, brain, biliary, endometrial)
Selumetinib	MEK1/2	Cancer (NSCLC. liver, melanoma, colorectal, thyroid, pancreatic, biliary)	Array BioPharma	Phase III (NSCLC) Phase II (liver, melanoma, colorectal, thyroid, pancreatic, biliary)
Binimetinib	MEK1/2	Cancer (melanoma, ovarian, peritoneal, colorectal, breast, pancreatic, esophageal, thyroid, hematological) Inflammation (rheumatoid arthritis) Metabolic disorders (cardiomyopathy)	Array BioPharma / Novartis	Phase III (cancer: melanoma, ovarian, peritoneal) Phase II (cancer: colorectal, breast, pancreatic, esophageal, thyroid, hematological) Phase II (inflammation: rheumatoid arthritis Phase II (CV: hypertrophic cardiomyopathy)
AEZS-136	PI3K and ERK1/2	Cancer (unspecified)	Aeterna Zentaris	Preclinical
FR-180204	ERK1/2	Rheumatoid arthritis	Astellas	No development reported

Note. See Refs. [4–6]. NSCLC, non-small cell lung carcinoma; FDA, U.S. Food and Drug Association; CV, cardiovascular; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase.

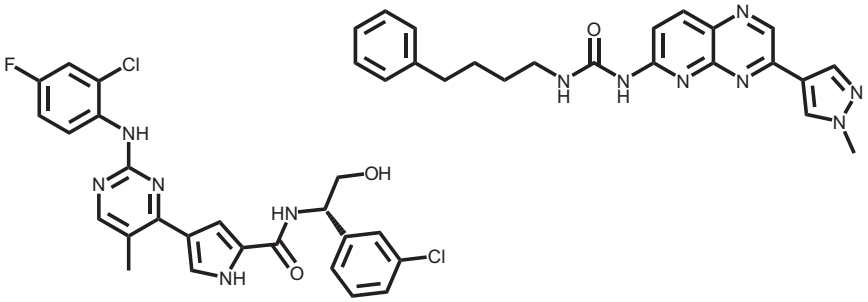

Fig.1. Two-dimensional structures of compound **1** (left) and compound **2** (right).

allowed us to quickly identify several slowly dissociating inhibitors for ERK2 with long and medium range residence times. Two of these compounds, **1** and **2** (Fig. 1), were further pursued for in-depth kinetic analysis against the active and inactive forms of ERK2. Our data show that although these two time-dependent inhibitors display similar K_i and k_{off} values when bound to inactive ERK2, only one compound, namely **1**, displays a higher potency and a much longer residence time when bound to the active form of the enzyme. The significance of these data is further discussed in this article in the context of kinetic efficiency and residence time.

Materials and methods

Materials

Active (i.e., phosphorylated) ERK2 is an N-terminal GST-tagged, human recombinant, full-length kinase (residues 1–360, 67.8 kDa, 97.4% purity estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE]) expressed in *Escherichia coli* and purchased from EMD Millipore (Darmstadt, Germany). Inactive (i.e., non-phosphorylated) ERK2 is a human recombinant, His-tagged (then cleaved), full-length kinase expressed in *E. coli* and purified in-house (residues 1–360, 41.6 kDa, >97% purity estimated by SDS–PAGE and liquid chromatography–mass spectrometry [LC–MS]). The Alexa 647 fluorescent-labeled active site probe and time-resolved fluorescence resonance energy transfer (TR–FRET) dilution buffer were purchased from Life Technologies (Carlsbad, CA, USA). The chemical formula of the active site probe is proprietary and was not disclosed by Life Technologies. Streptavidin–europium was purchased from Cisbio (San Diego, CA, USA). The mobility shift assay substrate (5-FAM-IPTSPITTTYFFFKKK) was purchased from CPC Scientific (Sunnyvale, CA, USA). NHS–PEG4–biotin used to generate biotinyl-ERK2 was purchased from Thermo Fisher (Waltham, MA, USA). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Compound **1** (aka VTX-11e) and compound **2**, previously published by Aronov and coworkers [12] and in patent WO2012/136691 A1, were synthesized in-house at Takeda California (San Diego, CA, USA). GraphPad Prism and Spotfire analysis software are commercialized by GraphPad Software (La Jolla, CA, USA) and TIBCO Spotfire Software (Boston, MA, USA). Unless stated otherwise, the kinetic studies listed in the "Methods" section were conducted using 50 mM Hepes buffer containing 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 0.01% Brij 35, and 10 mM $MgCl_2$ at pH 7.3 and room temperature. All of the reagent concentrations listed in the "Methods" section represent the final concentrations in the assay mixture.

Methods

General protocol for k_{off} and residence time determination in the presence of inactive ERK2

The determination of k_{off} with the inactive enzyme was conducted using a modified TR–FRET LanthaScreen binding assay.