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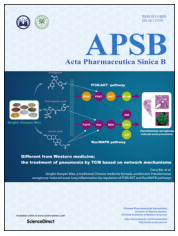

ORIGINAL ARTICLE

A high-throughput screening assay for eukaryotic elongation factor 2 kinase inhibitors

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Abstract Eukaryotic elongation factor 2 kinase (eEF2K) inhibitors may aid in the development of new therapeutic agents to combat cancer. Purified human eEF2K was obtained from an *Escherichia coli* expression system and a luminescence-based high-throughput screening (HTS) assay was developed using MH-1 peptide as the substrate. The luminescent readouts correlated with the amount of adenosine triphosphate remaining in the kinase reaction. This method was applied to a large-scale screening campaign against a diverse compound library and subsequent confirmation studies. Nine initial hits showing inhibitory activities on eEF2K were identified from 56,000 synthetic compounds during the HTS campaign, of which, five were chosen to test their effects in cancer cell lines.

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1. Introduction

Protein is the most important structural and functional macromolecule in the cell, therefore, the process of translation (also called protein synthesis), which translates a messenger RNA (mRNA) into a protein, is essential in this regard. Protein synthesis is usually divided into three main stages: initiation, elongation and termination[1]. The initial stage involves a variety of translation initiation factors that work coordinately to provide stringent regulatory mechanisms of translation. The elongation stage consumes a large amount of energy and amino acids within cells to synthesize new polypeptide chain, which will undergo further post-translational modification and fold into correct quaternary structure to function in the cell. Indeed, more than 99% of the energy and nutrients used in protein synthesis are consumed during elongation. Studies on translation termination mainly focus on abnormal signals and their effects on mRNA degradation while the understanding of other aspects of this step is rather limited[2].

Human eukaryotic elongation factor 2 (eEF2) is a member of the GTP-binding translation factor family that promotes the movement of ribosome along mRNA from one codon to the next[3,4]. During the elongation stage, eEF2 facilitates the translocation of peptidyl-tRNA from ribosome A site to P site. eEF2 can be phosphorylated and thus inactivated by human eEF2K[5]. The phosphorylation of eEF2 plays an important role in the regulation of protein synthesis. It was widely recognized that under stress conditions such as nutrient starvation, hypoxia or acidosis, the activity of eEF2K in tumor cells was upregulated which allows tumor cells to adapt and survive in such an adverse microenvironment. Thus, eEF2K has been proposed as a potential target for cancer therapy.

Among the human kinome, the majority (90%) of protein serine/threonine and tyrosine kinases share a similar architecture within their catalytic domain, so they are classified as conventional protein kinases (CPKs). The remainder (about 10%), which displays little sequence homology with CPKs, are named as atypical protein kinases (APKs). APKs and CPKs lack sequence identity, but share a related catalytic core[6] as well as a remarkably similar N-terminal lobe that predominantly folds into a curved β-sheet and contains the phosphate binding loop (P-loop). The interlobe cleft serves as ATP binding pocket and possesses conserved key catalytic residues[7]. Despite having similar ATP binding pockets, the spatial location of their conserved region GXGXXG is different. eEF2K is such an atypical Ser/Thr-protein kinase that it was also known as 'Ca^{2+}/CaM-kinase III'. Human eEF2K is composed of 725 amino acids.

It is well established that phosphorylation at Thr56 inactivates eEF2 thereby regulating protein synthesis at the elongation step of translation[8,9]. This mechanism appears crucial for cancer cell survival as it reduces energy and amino acid consumption to help them live through adverse conditions[10]. This point of view is supported by studies on cellular microenvironment[11] and metabolic stress[10] with breast cancer cells[12,13]. Since eEF2K is neither a conventional protein kinase nor has other known substrates, blockage of its action does not significantly affect normal biological processes. eEF2K inhibitors can become new drug candidates for cancer therapy.

It was thought that an eEF2 peptide (amino acid sequence: SARAGETRFTDTRKDE) containing the phosphorylation site was the substrate of eEF2K. When tested *in vitro*, however, it did not work efficiently. Scientists subsequently found two protein kinases MHCK (myosin heavy chain kinase from *Dictyostelium discoideum*) A and B, whose structures had considerable similarity to eEF2K, could phosphorylate a 16 amino acid peptide MH-1 (amino acid sequence: RKKFGESEKTKTKEFL) *in vitro*, an action that can be replicated by eEF2K[14]. Thus, MH-1 peptide has been used as a surrogate substrate for eEF2K.

In this study, we developed and validated a luminescence-based high-throughput screening (HTS) assay for the identification of eEF2K inhibitors by use of MH-1 peptide. Traditional method to detect the catalytic activities of protein kinase is to quantify the incorporation of radioactive γ-phosphate form [γ-^{32}P] adenosine triphosphate (ATP) into a peptide or protein substrate by using a scintillation counter. This is impracticable for HTS application. Immunoblotting assay can also be employed in screening, but the sample preparation is complex and costs time and money: it is therefore not suitable for HTS and could be readily replaced by fluorescence and luminescence approaches. MH-1 peptide substrates have been commercialized. For instance, ADP-Glo™ luminescent kinase assay kit can eliminate redundant ATP in the assay system and transform ADP into ATP effectively. This became the key technology used in this study.

2. Materials and methods

2.1. Reagents

A484954, eEF2K enzyme produced and purified from *Escherichia coli* according to the method previously described[15] and calmodulin were provided by Professor C. Proud at South Australia Health and Medical Research Institute. MH-1 peptide was purchased from China Peptides Co., Ltd. (Shanghai, China). EDTA, EGTA, $CaCl_2$ and $MgCl_2$ were bought from Shanghai Chemical Reagents Co., Ltd. (Shanghai, China). 3-(*N*-morpholino) propanesulfonic acid (MOPS), DMSO and β-mercaptoethanol (β-ME) were obtained from Sigma (St Louis, MO, USA). The assay plates were the products of PerkinElmer (Boston, MA, USA). ADP-Glo™ luminescent kinase assay kit was procured from Promega (Wisconsin, MA, USA). The anti-eEF2 and anti-phospho-eEF2 antibodies were purchased from Cell Signaling Technologies (Boston, MA, USA). L15 medium, fetal bovine serum, penicillin, streptomycin, sodium pyruvate, geneticin and 0.5% trypsin–EDTA were bought from Life Technologies (Carlsbad, CA, USA). Cancer cell lines used in this study were obtained from ATCC (Manassas, VA, USA).

2.2. Cell culture

MDA-MB-453 cells were maintained in L15 medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate and 0.5 mg/mL geneticin without CO_2. H1299 cells and HCT116 cells were maintained in RPMI-1640 medium and McCoy's 5a medium containing 10% FBS, respectively, 100 U/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate and 0.5 mg/mL geneticin in a 5% CO_2 incubator.

2.3. Compound library

The compound library used for the screening of eEF2K inhibitors is consisted of 56,000 pure synthetic compounds. The stock was

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