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Research paper

Design, synthesis and structure-activity relationship of a focused library of β-phenylalanine derivatives as novel eEF2K inhibitors with apoptosis-inducing mechanisms in breast cancer

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ABSTRACT

Eukaryotic elongation factor 2 kinase (eEF2K) is a Ca^{2+}/calmudulin-dependent protein kinase, belonging to a small family of an atypical Ser/Thr-protein kinase. eEF2K has been recently reported to be highly activated or overexpressed in many types of cancer; therefore, eEF2K would be regarded as a promising therapeutic target. In this study, we discovered a β-phenylalanine scaffold by virtual high-throughput screening, as well as designed and synthesized 46 derivatives with assessment of inhibition activity against eEF2K and cytotoxicity. After several rounds of kinase and anti-proliferative activity screening, we discovered an eEF2K inhibitor (**21l**) with best eEF2K enzymatic activity (IC_{50} of 5.5 μM) and anti-proliferative activity (MDA-MB-231 cells, IC_{50} of 12.6 μM, MDA-MB-436 cells, IC_{50} of 19.8 μM). Moreover, we found that **21l** could induce cell death via the apoptotic pathways in MDA-MB-231 and MDA-MB-436 cells. Subsequently, we evaluated its anti-tumor activity and apoptosis-inducing mechanisms *in vivo*. These results suggested that **21l** inhibited tumor growth by apoptosis in the xenograft mouse model of breast cancer (MDA-MB-231 and MDA-MB-436). Collectively, our results demonstrate a novel small-molecule inhibitor targeting eEF2K with mechanism of apoptosis and a therapeutic potential in breast cancer.

1. Introduction

Breast cancer (BC) is well known to be the most common malignancy among women, which is the world's second leading cause of cancer-related death [1,2]. It is a highly complex disease classified as five major subtypes with different biological features, levels of gene expressions and clinical behaviors [3]. Nowadays, principles of systemic therapies mainly are including chemotherapy, targeted therapy and endocrine therapy [4]. Therefore, more effective therapy strategies for better understanding of novel signaling pathways and molecular targets should be further provided and new anti-tumor targeted drugs should be explored afterwards.

Eukaryotic elongation factor-2 kinase (eEF2K) is a Ca2+/calmodulin-dependent protein kinase encoded by the eEF2K gene in human [5]. eEF2K can regulate the rate of peptide chain elongation to regulate protein synthesis by phosphorylating eEF2 (at Thr 56) or autophosphorylation [6–9]. Recently, accumulating studies have shown that eEF2K could promote tumor cell survival, proliferation, development, angiogenesis and resistance to chemotherapy [10–13]. Moreover, eEF2K inhibitors can be regarded as a potential strategy for malignant glioma and so on [14]. Nevertheless, there are no studies focusing on eEF2K inhibitors targeting breast cancer. Because the high expression level of eEF2K is frequently observed in different types of cancer, including breast cancer, it should be utilized as a potential therapeutic target for the treatment of breast cancer [15,16]. As mentioned above, novel eEF2K inhibitors bearing anti-proliferative effect on breast cancer should be deserved to further investigate.

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Recently, several eEF2k inhibitors have been developed (Fig. 1). TS-2 was the first identified selective eEF2K inhibitor with IC_{50} of 0.36 μM, which inhibited ATP or eEF2 binding to eEF-2K in a competitive or non-competitive manner, respectively. TS-2 also decreased phospho-eEF2 protein level without changing the total eEF2 protein level [17]. TX-1918 was identified as a non-selective eEF2K inhibitor with IC_{50} of 0.44 μM. In addition, TX-1918 also showed certain inhibitory activity against multiple tyrosine kinases, such as PKA, PKC and Src-K. However, TX-1918 did not show antitumor activity in several cancer cells [18]. A484954, a pyrido [2,3-*d*]pyrimidine-2,4-dione derivative, exhibited potent eEF2k inhibitory activity *in vitro* recombinant kinase inhibition assays with IC_{50} of 0.42 μM A484954 could significantly reduce eEF-2K activity in MDA-MB-231 breast cancer cells. Regretfully, A484954 showed almost no cytotoxicity to lose drugable potential, which was utilized as a positive eEF2K inhibitor *in vitro* [19]. NH125 has been widely used as an eEF2K specific inhibitor with IC_{50} of 60 nM *in vitro* [20]. More recently, it has been reported that NH125 cannot specifically inhibit eEF2K, which inhibits tumor growth by up-regulating the phosphorylation level of eEF2 [21]. Some natural products were reported to inhibit eEF2K activity *in vitro*, such as rottlerin [22], GA [23]. Collectedly, the development of eEF2K specific inhibitors is still in its infancy. Thus, it is considered as a promising avenue to develop novel and special-small molecule inhibitors targeting eEF2K with potent inhibitory activity *in vivo* and *in vitro* for breast cancer therapy. In this study, we discovered a novel small-molecule inhibitor targeting eEF2K with mechanism of apoptosis *in vitro* and *in vivo*, which would provide a promising strategy in cancer treatment.

2. Results and discussion

2.1. The discovery of potential eEF2K inhibitors by virtual screening and western blot analysis

Since the crystal structure of eEF2K has not been resolved, we build the model of kinase domain of eEF2K through the Swiss-model web portal server [17], a web server for protein modeling. Myosin heavy chain kinase A (MHCK A) was selected as the template, the sequence identity was 41.74%, the coverage is 0.3 other detail information was seen Figure S1. Subsequently, the model was refined and estimated by PROCHECK tool. These results indicated that the model was of good quality to use (Figure S2). Three-step molecular docking was employed to screen lead compounds of an eEF2K inhibitor from ChemBridge CORE library modified by Lipinski's Rule of Five by Discovery studio 3.5 (Fig. 2A). Top 100 hits were selected by LibDock protocol in the first step. Subsequently, Top10 hits were further screened by CDOCKER protocol (Fig. 2B).

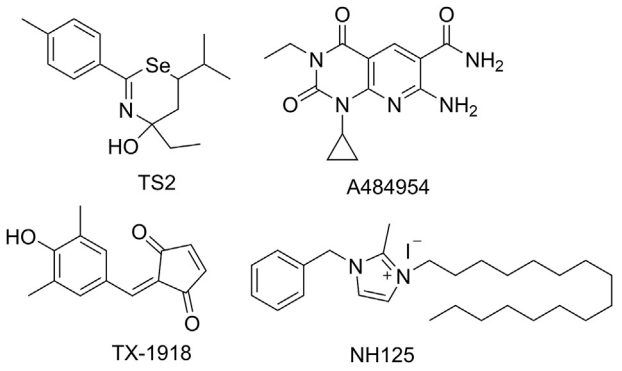

Fig. 1. Structures of several eEF2K inhibitors.

Since mutation of ser78 to a non-phosphorylatable alanine residue decreased eEF2K activity, thus we used *p*-eEF2K (ser78) to monitor the eEF2K activity [24]. Subsequently, we tested the inhibitory activity of 10 hits against eEF2K by determining the expression levels of eEF2K and *p*-eEF2K (ser78). The result indicated that compound **4, 7,** and **9** showed certain inhibitory activities against eEF2K (Fig. 3). And the inhibitory activity of compound **9** was showed little less than compound **4** (A484954). Hence, compound **9** was selected as the candidate for further optimization as the eEF2K inhibitors.

2.2. Chemistry

The synthesis of desired compounds was obtained by the route as (Scheme 1). The intermediate **2** was prepared by Knoevenagel reaction commercially available substituted benzaldehyde **1** with malonic acid in present of ammonium formate. The intermediate **2** was treated with HCl saturated methanol solution to give the intermediate **3**. Treatment of **3** with appropriate acyl chlorides or sulfonyl chlorides and triethylamine in dichloromethane afforded the acylation intermediates. The desired compounds were afforded by hydrolysis with sodium hydroxide solution.

2.3. SARs of compounds

In order to increasing the activity against eEF2K of lead compound by the reasonable optimization, a focused library of 46 derivatives was designed and synthesized. First, we introduced a series of substituent groups to replace the 4-fluorophenyl to give compounds **18b-e**. The results reveal that compounds **18b-d** showed a loss of activity, while compound **18e** with 2, 4-dichorophenyl showed a slightly improved activity against eEF2k with IC_{50} of 29.3 μM and a weak anti-proliferative activity with IC_{50} of 67.2 μM in MDA-MB-231 cells. Subsequently, the sulfamide group was used as the linker with diverse substituted aromatic rings, leading to compounds **18f-n**. The enzyme activity indicated that these compounds bearing para electron-withdrawing groups showed more potent activity, such as compound **18h**, **18k** and **18l**. To further explore the chemical space for activity improvement, we established different substituted scaffolds, including 4-*t*-butyl, 2-fluoro and 2, 4-dichloro group, leading to compounds **19a-k**, **20a-h** and **21a-m**. According to the biological activity results (Table 1), compound **21l** showed best eEF2k enzymatic activity (IC_{50} of 5.5 μM), and anti-proliferative activity (MDA-MB-231 cells, IC_{50} of 12.6 μM, MDA-MB-436 cells, IC_{50} of 19.8 μM). Intriguingly, the di-Cl compound **21l** was better than the parent unsubstituted compound 18k in activity. We performed the molecular docking of eEF2K with **21l** and **18k,** respectively. The result revealed that the **18k** presented a different pose from **21l**. These poses couldn't form the two key hydrogen bonds in the kinase hinge observed in **21l**, which might provide a partial explanation for activity difference (Figure S3). To provide insights into the mechanism of small molecule inhibitor on eEF2k function, we selected **21l** to further evaluation of anticancer activity *in vitro*.

2.4. Molecular docking and molecular dynamic (MD) stimulation of *21l* with eEF2K

To explore the potential interaction mode between **21l** and eEF2K, a molecular modeling study was performed (Fig. 4B and C). The results showed that the carboxyl of **21l** could form a hydrogen bond with the side chain of Try236 and an additional hydrogen bond was initiated between cyanogroup of **21l** and Ile232, the two key residues (Try236, Ile232) located in the kinase hinge of ATP binding site. The sulfamide linker could form a key hydrogen bond interaction with the side chain of Arg140. In addition, the cyano-