AMINOPYRIDINE- AND AMINOPYRIMIDINE-BASED SERINE/THREONINE PROTEIN KINASE INHIBITORS ARE DRUG CANDIDATES FOR TREATING DRUG-RESISTANT TUBERCULOSIS

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Tuberculosis (TB) is the world's deadliest bacterial infection. Its causative agent *Mycobacterium tuberculosis* evolves into rapidly spreading multidrug-resistant and extensively drug-resistant (MDR and XDR) strains, which complicates the treatment. Therefore, the use of novel target-specific chemical compounds is crucial for the development of effective antituberculosis agents. Serine/threonine protein kinases (STPKs) of *M. tuberculosis* are currently considered as attractive drug targets. In turn, aminopyridines and aminopyrimidines that have not been used for TB treatment so far exhibit inhibitory activity towards STPKs. In this study we screened 192 aminopyridine- and aminopyrimidine-based compounds using the M*ycobacterium smegmatis aphVIII+* test system designed to screen for active STPKs inhibitors. First, we selected 53 compounds with subinhibiting concentrations of up to 100 nmol/disk. Of them, 22 showed STPKs-inhibiting activity in the test system, which was confirmed *in vitro* on the *M. tuberculosis* PknA protein with a maximum of 26.9 ± 6.1 %. Toxicity testing was performed *in vitro* on human embryo fibroblasts using the MTT-assay. Ultimately, 3 relatively active and relatively non-toxic STPKs inhibitors were selected for further research as drug candidates for MDR-TB treatment.

Keywords: tuberculosis, multidrug resistance, serine/threonine protein kinases, aminopyridines, aminopyrimidines, inhibitors, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *aphVIII*

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ИНГИБИТОРЫ СЕРИН-ТРЕОНИНОВЫХ ПРОТЕИНКИНАЗ КЛАССОВ АМИНОПИРИДИНОВ И АМИНОПИРИМИДИНОВ — КАНДИДАТЫ В ПРЕПАРАТЫ ДЛЯ ЛЕЧЕНИЯ ЛЕКАРСТВЕННО-УСТОЙЧИВЫХ ФОРМ ТУБЕРКУЛЕЗА

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Туберкулез — самая смертоносная бактериальная инфекция из известных человеку, при этом ее лечение осложнено появлением и быстрым распространением штаммов возбудителя, *Mycobacterium tuberculosis*, с множественной и широкой лекарственной устойчивостью (МЛУ и ШЛУ). В результате главным требованием к разрабатываемым противотуберкулезным препаратам является использование новых классов химических соединений, поражающих новые биомишени. Серин-треониновые протеинкиназы (СТПК) — перспективные мишени, а аминопиридины и аминопиримидины, ранее не применявшиеся в качестве противотуберкулезных препаратов, имеют предсказанную активность в отношении СТПК. В данной работе в тест-системе *Mycobacterium smegmatis aphVIII+*, предназначенной для отбора ингибиторов СТПК на клеточном уровне, был проведен скрининг 192 соединений двух указанных классов. Сначала отобрали 53 соединения с субингибирующей концентрацией до 100 нмоль/диск. Из них 22 соединения проявили активность в тест-системе как ингибиторы СТПК, которая была подтверждена *in vitro* на белке PknA *M. tuberculosis* (наивысшее значение показателя ингибирования — 26,9 ± 6,1 %). Также отобранные соединения тестировали на токсичность *in vitro* на клетках фибробластов эмбриона человека с использованием МТТ-теста. В результате для дальнейших исследований в качестве новых препаратов для борьбы с МЛУ-туберкулезом были отобраны 3 ингибитора СТПК с относительно высокой активностью и относительно низкой токсичностью.

Ключевые слова: туберкулез, множественная лекарственная устойчивость, серин-треониновые протеинкиназы, аминопиридины, аминопиримидины, ингибиторы, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *aphVIII*

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At present, tuberculosis (TB) is one of the deadliest infections accounting for 1.4 million deaths and 10.4 incident cases every year [1]. Its treatment is seriously complicated by increasingly emerging multidrug-resistant (MDR) strains of its causative agent *Mycobacterium tuberculosis*, i. e. strains resistant to the most effective and lowly toxic first-line drugs rifampicin and isoniazid [2]. This necessitates the use of more expensive and toxic second-line drugs [3]. A particular case of MDR is extensive drug-resistance (XDR), i. e. MDR aggravated by resistance to at least one of fluoroquinolone antibiotics and any of the second-line injectables (amikacin, kanamycin or capreomycin) [3]. Recently, there have been reports in Iran, India and South Africa of *M. tuberculosis* strains resistant to all known first- and second-line drugs (referred to as totally drug-resistant forms) [4–6]. Russia and former Soviet republics are among countries with the highest MDR-TB burden [7]: MDR is observed in one in every five incident cases, and every second patient with previously treated TB has MDR [1].

The era of antibiotics, i.e. the last 50 or 60 year, is marked directional selection of drug-resistant *M. tuberculosis* strains. Given that normal rate of random mutations is 10^{-6}–10^{-8} per cell division, it should be highly improbable for MDR to develop, considering the use of combination therapy. However, there are a number of factors that contribute to MDR development: monotherapy resulted from poor drug supply, inadequate or ineffective chemotherapy regimens, and patient's non-compliance with the treatment [8]. Repeated and prolonged use of the same drugs also has its impact: bedaquiline is the first new anti-TB drug introduced in clinical routine over the past 40 years [9].

Advances in TB treatment depend on the discovery of new drugs capable of affect new biotargets to circumvent current resistance mechanisms. Serine/threonine protein kinases (STPKs) are attractive targets as they are universal regulators of the cell cycle in pro- and eukaryotes. In particular, these enzymes regulate growth, cell division, virulence, persistence, and intrinsic antibiotic resistance in mycobacteria [10–14]. An ability to selectively inhibit STPKs has been shown for aminopyridine- and aminopyrimidine-based compounds [15]; they have not been used for TB treatment before, therefore there are no mutant strains with resistance to these compounds.

Previously we have developed and validated a *Mycobacterium smegmatis aphVIII+* test system aimed to screen for active inhibitors of mycobacterial STPKs, in particular PknA of *M. tuberculosis* [16]. This work aimed to select active STPK inhibitors from a number of aminopyridine- and aminopyrimidine-based compounds as potential drugs candidates for MDR and XDR-TB treatment.

This article was crafted from a PhD dissertation in biology defended by one of the authors in December, 2016 [17]. The results presented in the dissertation have not been previously published elsewhere, and the authors believe they are quite important and should be shared with the scientific community.

METHODS

Bacterial strains and growth conditions

Strains used in this work were *Escherichia coli* BL21 (DE3) pLysS and *M. smegmatis mc*^{2} 155 *E. coli* were cultured in LB media (Amresco, USA); *M. smegmatis* were cultured in Lemco-Tw media (5 g/l Lemco Powder, 5 g/l NaCl, 5 g/l bacto peptone, 0.05 % Tween-80). Cultures in liquid media were incubated in the Multitron incubator shaker (Infors HT, Switzerland) at 37 °C and 250 rpm. Solid culture media contained 2.0 % agar. The culture medium used in the test system was M290 Soyabean Casein Digest Agar by HiMedia, India.

Protocol for testing compounds in M. smegmatis aphVIII+ test system

M. smegmatis aphVIII+ culture was diluted 1 : 9 : 10 (culture : water : M290 medium) and seeded over the base agar layer on Petri dishes. The culture medium was supplemented with 50 μg/ml of hygromycin and 10 ng/ml tetracycline. The dishes were allowed to dry; then paper discs were placed inside the dishes, impregnated with either a studied compound or kanamycin or a combination of both. The dishes were then incubated at 37 °C until the bacterial lawn was formed. Then growth inhibition halos were measured. All experiments were conducted in 3 to 5 replicates [16].

Purification of M. tuberculosis PknA and AphVIII proteins

Synthesis of the *M. tuberculosis pknA* gene was performed by Evrogen (Russia). For more efficient expression in *E. coli*, the gene was codon-optimized. *pknA* was synthesized and cloned into pET-32a expressing vector. Plasmid DNAs containing *pknA* and *aphVIII* (pET16b-aphVIII) genes [18] were transformed into the *E. coli* BL21 (DE3) pLysS strain by calcium chloride–mediated transformation [19]. Overnight expression of the gene was induced by 1mM isopropyl-β-D-thiogalactopyranoside (IPTG; Anatrace, USA). Proteins were purified using the Ni-NTA Fast Start Kit (Qiagen, USA).

In vitro kinase assay

Inhibiting activity of aminopyridine- and aminopyrimidine-based compounds against PknA and AphVIII was assessed by the kinase reaction using Kinase-Glo Plus Luminiscent Kinase Assay Kit (Promega, USA) and the Biomek 3000 workstation (Beckman Coulter, USA); the technique applied was previously described by Baki et al. [20]. Substrate-level phosphorylation was estimated indirectly by measuring luminescence of residual ATP. Oligopeptide IVDAELTGEIPII was used as a PknA substrate, and kanamycin was used as an AphVIII substrate. Reaction was performed overnight in the working solution containing 15 mM HEPES (pH 7.4), 20 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.02 % Tween-20, and 0.1 mg/ml BSA.

The reaction mix (45 μl) for PknA kinase assay contained 3 μg protein, 5 μM ATP, and 50 μg substrate.

The reaction mix (45 μl) for AphVIII kinase assay contained 50 ng protein, 10 μM ATP, and 5 μg substrate.

Cytotoxicity of compounds

Cytotoxicity of aminopyridine- and aminopyrimidine-based compounds was estimated by the MTT assay using human embryonic fibroblasts derived from fetal skin and muscles (HEF-4). Cell viability was estimated by color development accompanying tetrazole reduction to formazon by mitochondrial dehydrogenases. Measurements were recorded on the Beckman Coulter DTX 880 Multimode Detector (a plate reader by Beckman Coulter, USA) at excitation wavelength of 595 nm. Absorbance from the wells of cells cultured with the control media was taken as 100 % [21].

RESULTS

Screening for active STPK inhibitors in the M. smegmatis aphVIII+ test system

Using the disc method, we selected a number of aminopyridine- and aminopyrimidine-based compounds that exhibited STPK-inhibiting activity in the *M. smegmatis aphVIII+* test system validated earlier.

The test system employs the following principle: STPK MSMEG_5513 phosphorylates APHVIII protein in *M. smegmatis* cells enhancing their resistance to kanamycin. After an MSMEG_5513 inhibitor is added to the system, APHVIII phosphorylation decreases and the activity of the enzyme diminishes reducing resistance of bacterial cells to kanamycin. The intrinsic antimicrobial activity of the inhibitor is determined by its ability to inhibit another STPK: MSMEG_0030 (ortholog of *M. tuberculosis* PknA), a vitally important protein for mycobacteria, and possibly some other targets. In our experiment reduced resistance to kanamycin was expressed as a larger growth inhibition halo around the disc impregnated with a combination of kanamycin and an active STPK inhibitor, compared to the halo around the disk treated with kanamycin only (Fig. 1) [16].

One of the selection criteria for compounds in the *M. smegmatis aphVIII+* test system was their subinhibiting concentration. For 53 compounds it was as low as 100 nmol/disc or less, while the rest of the compounds did not have any antibacterial effect on *M. smegmatis* at this concentration. The selected compounds were tested in the test system. STPK inhibitor LCTA-1389 (11b) [22, 23] was used as a positive control. BisV, the inactive analog of standard STPK inhibitors from the indolyl maleimide family [24], was used as a negative control.

For 22 studied compounds, in solid media a bacterial growth inhibition halo around the discs treated with both kanamycin and an STPK inhibitor was significantly larger than around the discs treated with kanamycin only. The following "hit" compounds were selected for further testing of their potential as mycobacterial STPKs inhibitors (Fig. 2): 1f8, 1g8, 1e11, 1g11, 1h11, 1a12, 1c8, 2f4, 2c3, 2c6, 2a3, 2a4, 2a7, 2h11, 2h12, 2d3, 2d11, 2b4, 2b5, 2e12, 2g12, 2h12.

Inhibiting activity of selected compounds against M. tuberculosis PknA protein in vitro

The above listed compounds were tested for their ability to inhibit *M. tuberculosis* STPK PknA *in vitro* at concentrations of 200 μM (inhibitor : target molar ratio of 154 : 1). LCTA-1389

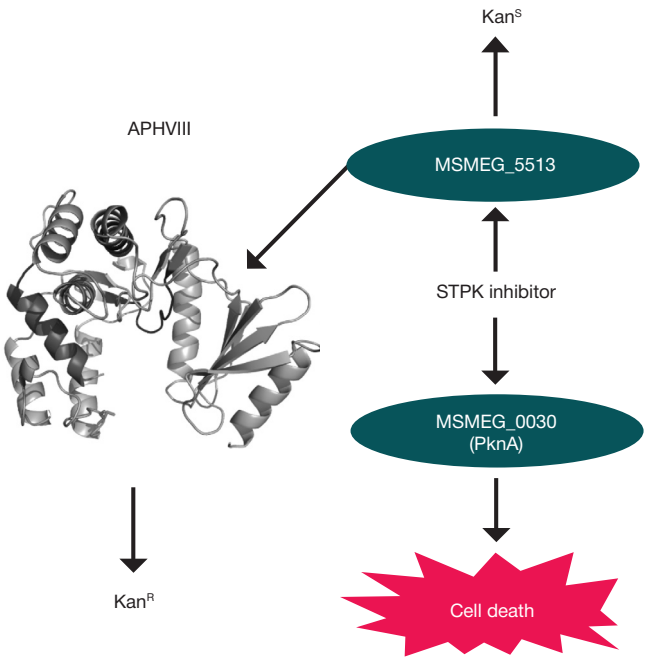

Fig. 1. Principle of *Mycobacterium smegmatis aphVIII+* test system

Kan^R — increased resistance to kanamycin, Kan^S — reduced resistance to kanamycin, STPK — serine/threonine protein kinase. Detailed description is provided in the article.

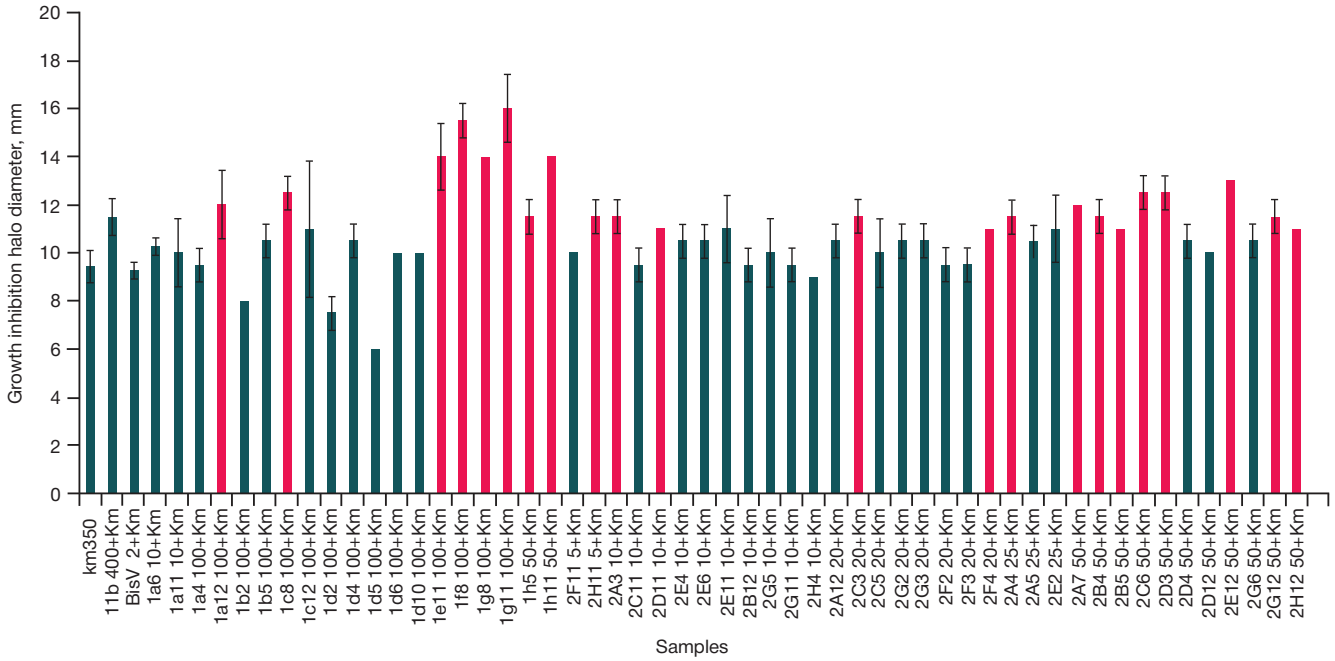

Fig. 2. Diameter of bacterial growth inhibition halos around discs impregnated with aminopyridine- and aminopyrimidine-based compounds

The compounds were tested in the *Mycobacterium smegmatis aphVIII+* test system at subinhibitory concentrations (nmol/disc; specified near the sample name) that do not produce a bacterial growth inhibition halo. Error bars represent standard deviation. Selected compounds are shown in red.

(11b) and BisV were used as positive and negative controls, respectively. Results are shown in Fig. 3. All compounds exhibited inhibiting activity similar to that of a positive control or higher, as in the case of two compounds: 1H11 (26.9 ± 6.1 %) and 2G12 (23.2 ± 2.0 %).

All of these compounds were tested at the same molar ratio (inhibitor : target) for their ability to inhibit phosphotransferase activity of APHVIII *in vitro* to make sure their activity in *M. smegmatis aphVIII*+ system was selective. Results were negative in all cases, i. e. activity of the compounds in the test system was determined by their ability to inhibit STPKs of *M. smegmatis* only and not the APHVIII protein.

Cytotoxicity of selected compounds

Cytotoxicity of the selected compounds was tested on HEF-4 cells. Based on the results, the compounds were divided into three groups: highly toxic (< 10 μg/ml; 1F8, 1G11, 2D11, 2F4, 2C3, 2A3, 2H11); moderately toxic (10–50 μg/ml; 1E11, 1G8, 1H11, 2D3, 2E12, 2G12, 2A4, 2A7); and low toxic (> 50 μg/ml; 2C6).

DISCUSSION

In the age of emerging antibiotic resistance of *M. tuberculosis* strains, effective drugs for TB treatment must meet two basic requirements: a novel mechanism of action and low toxicity.

In the past 15 years, development of antibacterial drugs, including antimycobacterial agents, was based on the biochemical targeted selection of compounds inhibiting essential bacterial enzymes. This concept had certain limitations when applied to *M. tuberculosis*: the overwhelming number of the compounds that were supposed to have anti-TB potential did not have any effect on the bacterial cell for a number of reasons, such as poor permeability of the bacterial cell wall [25]. So researchers went back to using a rapidly growing *M. smegmatis* in screening tests because its cellular wall is similar to that of *M. tuberculosis* in terms of permeability. This is how bedaquiline was discovered [26]. However, this approach dictates a need for further validation of a drug target [27].

The *M. smegmatis aphVIII*+ test system was designed to select candidate compounds based on their antimycobacterial effect and target specificity [16]. The latter was confirmed *in vitro* for the compounds selected in our experiment. However, a limitation of our study is the lack of possibility to determine a half maximal inhibitory concentration IC_{50}, which might be related to the low activity of the purified thioredoxin fusion PknA protein. A large amount of protein in the reaction mix dictated a need for the analysis of maximal soluble concentrations of the tested compounds. However, the inhibiting activity of the selected compounds that was similar or even superior (1H11 and 2G12) to that of the positive control [22, 23] and a previously demonstrated inhibiting activity of aminopyridine- and aminopyrimidine-based compounds against STPKs [15] allow us to hypothesize that these compounds may be used as effective STPK inhibitors.

Based on screening results, we have selected three compounds as potential drug candidates that exhibited the highest activity in the *M. smegmatis aphVIII*+ test system, on the PknA protein *in vitro*, and the lowest toxicity against human cell culture (Fig. 4). The most active compounds 1H11 and 2G12 were classified as moderately toxic. However, it should be noted that our screening was only the first step in the selection of "hits", which could be further optimized to enhance their effect or reduce toxicity. Their activity must be tested on *M. tuberculosis*; acute and chronic toxicity must be assessed *in vivo*. These compounds must also be tested on human STPKs to make sure their activity is selective.

CONCLUSIONS

We have demonstrated feasibility of developing a new aminopyridine- and aminopyrimidine-based drugs for MDR and XDR TB treatment. Further tests of the selected "hits" (1E11, 2C6, 2G12) are required to assess and optimize their antimycobacterial activity against *M. tuberculosis* and reduce toxicity.

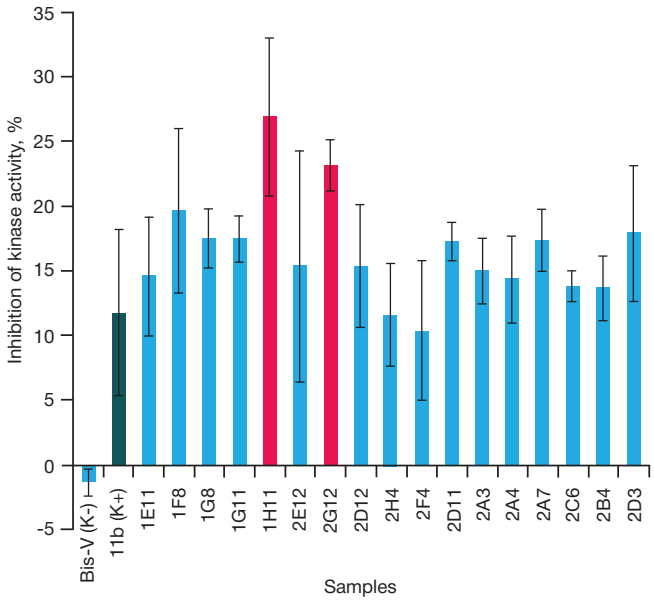

Fig. 3. Inhibition of phosphorylating activity of *Mycobacterium tuberculosis* PknA *in vitro* by compounds selected by the *Mycobacterium smegmatis aphVIII*+ test system

Error bars represent standard deviation. Positive control is shown in green. The most active compounds are shown in red.

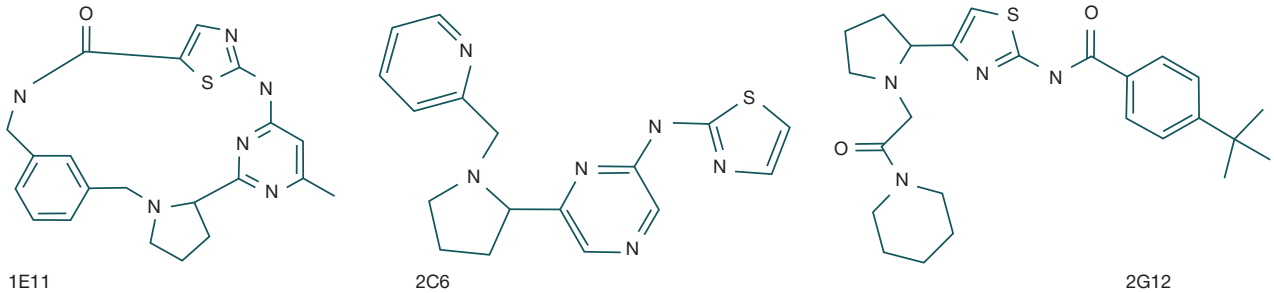

Fig. 4. Chemical structures of aminopyridine- and aminopyrimidine-based compounds selected as potential antituberculosis agents

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