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MOLECULAR SCREENING OF PROSPECTIVE CANDIDATES FOR TRPA$_1$ ION CHANNEL SELECTIVE ANTAGONISTS

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Abstract: TRPA$_1$ is an ankyrin receptor of TRP family. It is mostly expressed in the small-diameter nociceptors with cell bodies located in the dorsal roots, as well as in trigeminal, nodose and jugular ganglia. Recently, more and more information has been found in the literature on the role of TRPA$_1$ in the realization of cold and pain sensitivity, and also in the formation and maintenance of inflammation. Subject to these data there is an increasing interest in finding and studying pharmacological agents able to selectively block the TRPA$_1$ receptors and thereby reduce the severity of pain and inflammation.

Using the molecular modeling techniques, we analyzed spectra of biological activity of a series of promising candidates for selective antagonists of TRPA$_1$ ion channel in order to find potentially active compounds against pain and inflammation.

Substances that showed in high throughput screening the percentage of the cellular calcium response inhibition above 50% - 3*SD maximum and its own agonistic activity less than 10% + 3*SD minimum were identified as hits. The value IC50 for hits was determined immediately after re-testing at 10 μM concentration and repeatedly after the determination of the leading chemical series.

As the result of the studies, the biologically active molecules of leading chemical series have been confirmed. The research was partially supported by the grant of the President of the Russian Federation №MD-4711.2015.7 and МК-6135.2016.4.

Key words: TRPion channel**;** TRPA$_1$; pain; nociception; inflammation; molecular screening; hits.

Over the past 20 years there has been a revival of interest in the fundamental mechanisms of nociception [1, 2]. Recent advances in immunohistochemistry, neuropharmacology and neurophysiology contributed to new discoveries in the field of physiology and pathophysiology of pain. The conducted studies resulted in the discovery of new receptors and mechanisms of pain perception. The obtained information ensured the opportunity to create and apply new tools and methods for more effective and safe control over pain [3]. One of the modern methods of pain treatment is the use of various substances that can selectively block the receptors that directly perceive both pain stimuli and

inflammatory mediators. One of these receptors is an ankyrin receptor $TRPA_1$.

The $TRPA_1$ is mostly expressed in the small-diameter nociceptors with cell bodies located in the dorsal roots, as well as in trigeminal, nodose and jugular ganglia [1, 2, 4]. Some authors argue, according to the results of their research, for the increasing $TRPA_1$ expression in pathological conditions such as inflammation, neuropathy [5, 6, 7], by virtue whereof there has been an increase in interest in the $TRPA_1$ as the receptor of pain stimuli [8, 9].

Objective of the study was to confirm the activity of hits of the leading chemical series, obtained from high-throughput screening of a focused library.

Materials and research methods. Embryonic human liver epithelial cells exogenously expressing the IPTG (isopropyl-β-D-1-thiogalactopyranoside)-induced $TRPA_1$ ion channel, were suspended in culture medium with 1 mM IPTG so as to obtain a concentration 8x105 cells/ml, which would amount 20,000 cells per well. The resulting cell suspension was placed in a 384-well culture experimental dies with optical bottom with the use of automatic robotized stations Biomek FX or Biomek NX, 25 µg suspension in each well. 384-well dies were centrifuged at 200xg for 5 minutes. Further experimental dies with the cell suspension were transferred to an incubator at 37°C, 5% CO2. On the day of the experiment cells were immersed in Ca2+-sensitive dye, prepared by the manufacturer's instructions. Experimental dies filled with Ca2+-sensitive dye were incubated for 1 hour at a room temperature in a dark place. Further step was screening with FLIPR (Robotic High-Throughput Cellular Calcium Screening System).

The $TRPA_1$ ion channel was stimulated with agonist at EC80 concentration, which was 30 µM. For the inhibition of stimulated calcium response, we used the maximum inhibitory concentration of a control antagonist IC100, equal to 6 µM. FLIPR was turned on 30 minutes prior to the experiment to preheat its laser. We performed a signal test and adjusted the laser so as the base signal level was equal to 10,000 relative fluorescence units.

To detect agonist activity of test substances 12.5 µl substance was added to each well of 384-well die according to the die scheme. Further, the experimental dies were screened with FLIPR. After incubation, 12.5 µl $TRPA_1$ ion channel agonist and allyl isothiocyanate (AITC) were added for 11 minutes to each well of 384-well experimental die, and the second FLIPR screening immediately started to determine the antagonists.

The dependence of antagonist activity on concentration was analyzed for substances. IC50 value was determined from the obtained relation. The experimental data were analyzed with GraphPad Prizm (GraphPad Software, Inc., San Diego, CA). For the construction of concentration dependence the following equation was chosen: Y=Bottom curve plateau + (Upper plateau-Lower Plateau)/(1+10^((LogIC50-X)*Curve slope)).

Results of the study. The obtained results show that all previously discovered hits of leading chemical series have confirmed their activity towards the $TRPA_1$ ion channel (Table 1, Figure 1-3).

Table 1

Summary table with IC50 data for verifiable hits

Substance	IC50, µM	Comments
3455-3246	36.76	
3455-3273	11.82	
3455-3281	22.51	
3455-3320	13.22	partial antagonist
4606-1800	13.56	
5628-0670	12.81	
6040-4753	7.439	partial antagonist
E002-2494	0.9008	
E002-2495	3.747	
E002-2498	3.039	
G857-0153	7.6	
G721-0484	9.623	partial antagonist
C276-1460	46.74	

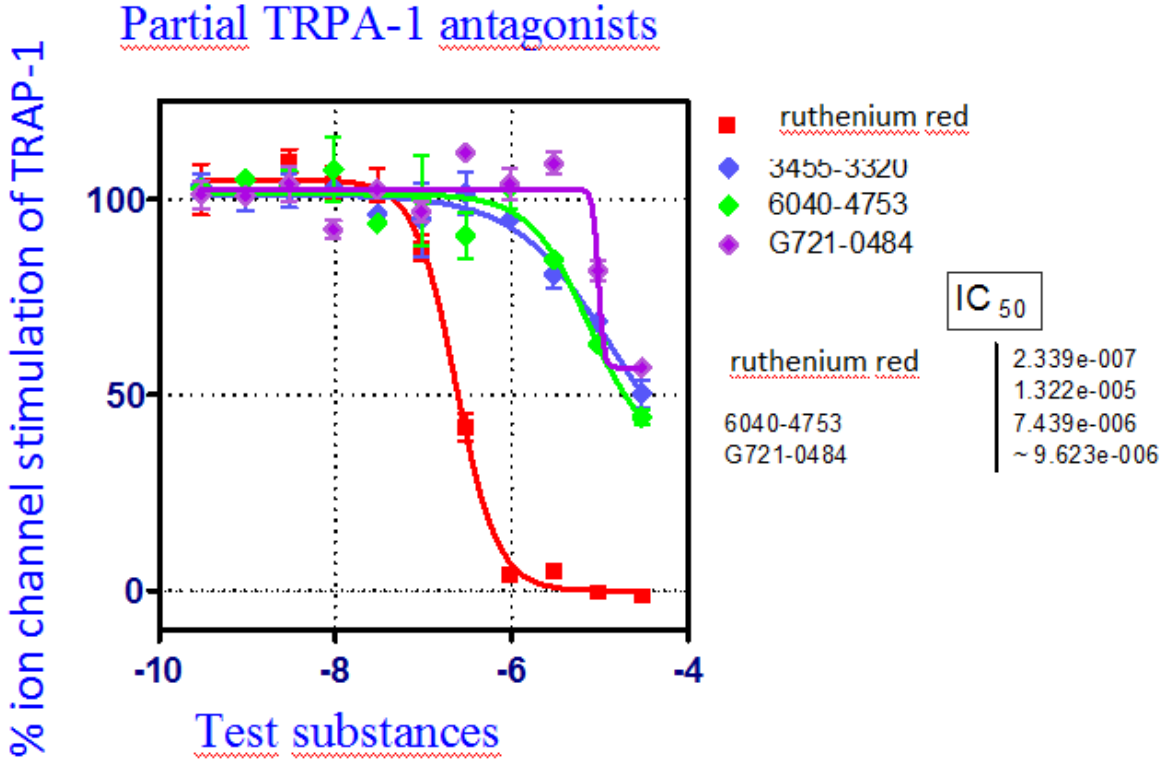

*Figure 1.*The concentration dependence of antagonist activity on the concentration for verifiable hits and control antagonist RR. Each concentration point is the average of two repetitions. Partial antagonists

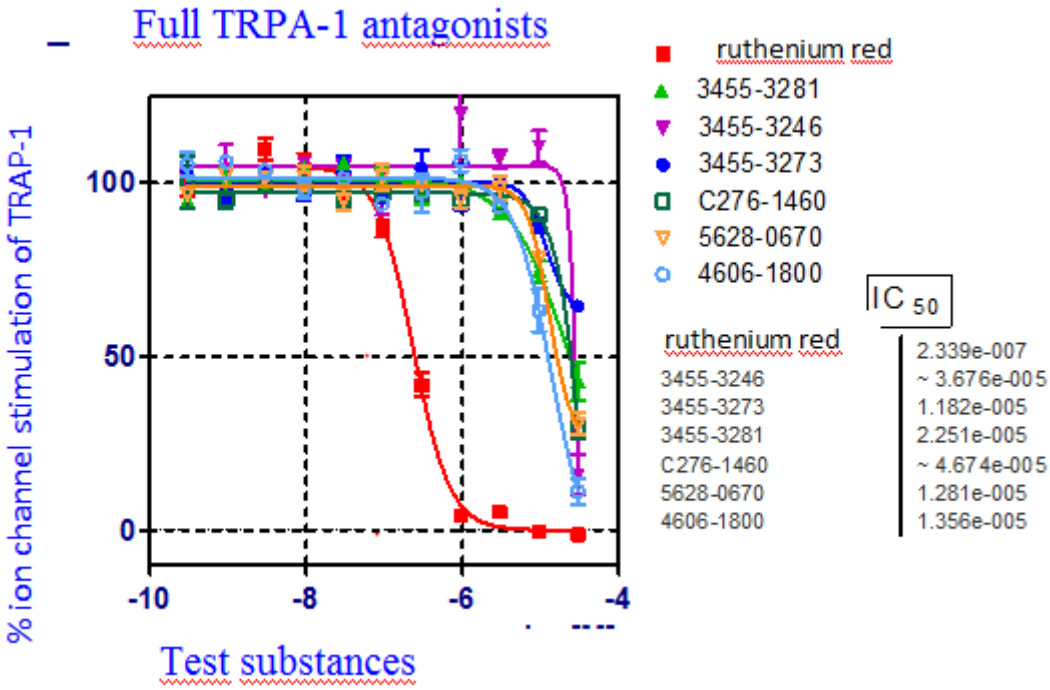

*Figure 2.*The concentration dependence of antagonist activity on the concentration for verifiable hits and control antagonist RR. Each concentration point is the average of two repetitions. Full antagonists

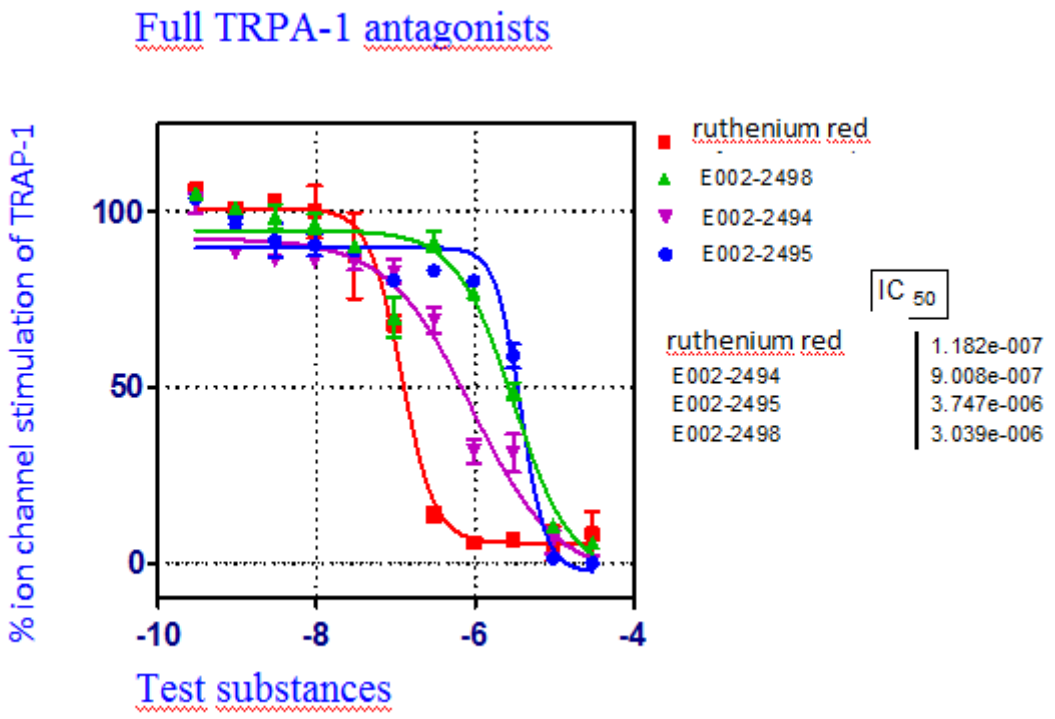

*Figure 3.*The concentration dependence of antagonist activity on the concentration for verifiable hits and control antagonist RR. Each concentration point is the average of two repetitions. Full antagonists

Summary. As a result of molecular screening of biologically active molecules- candidates for selective antagonists of $TRPA_1$ ion channel, it was found that all investigated hits of the leading chemical series confirmed their activity. Thus, these molecules can be recommended for further studies of their activity in the tests in vivo in laboratory animals.

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