In vitro screening of major neurotransmitter systems possibly involved in the mechanism of action of antibodies to S100 protein in released-active form

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Abstract: Experimentally and clinically, it was shown that released-active form of antibodies to S100 protein (RAF of Abs to S100) exerts a wide range of pharmacological activities: anxiolytic, antiasthenic, antiaggressive, stress-protective, anhydropic, antiischemic, neuroprotective, and nootropic. The purpose of this study was to determine the influence of RAF of Abs to S100 on major neurotransmitter systems (serotoninergic, GABAergic, dopaminergic, and on sigma receptors as well) which are possibly involved in its mechanism of pharmacological activity. Radioligand binding assays were used for assessment of the drug influence on ligand–receptor interaction. [35S]GTPγS binding assay, cyclic adenosine monophosphate HTRF™, cellular dielectric spectroscopy assays, and assays based on measurement of intracellular concentration of Ca²⁺ ions were used for assessment of agonist or antagonist properties of the drug toward receptors. RAF of Abs to S100 increased radioligand binding to 5-HT₁F, 5-HT₂B, 5-HT₂C, 5-HT₃, and to D₃ receptors by 142.0%, 131.9%, 149.3%, 120.7%, and 126.3%, respectively. Also, the drug significantly inhibited specific binding of radioligands to GABA_B₁A/B₂ receptors by 25.8%, and to both native and recombinant human sigma receptors by 75.3% and 40.32%, respectively. In the functional assays, it was shown that the drug exerted antagonism at 5-HT₁B, D₃, and GABA_B₁A/B₂ receptors inhibiting agonist-induced responses by 23.24%, 32.76%, and 30.2%, respectively. On the contrary, the drug exerted an agonist effect at 5-HT₁A receptors enhancing receptor functional activity by 28.0%. The pharmacological profiling of RAF of Abs to S100 among 27 receptor provides evidence for drug-related modification of major neurotransmitter systems.

Keywords: dopamine agent, released-activity, serotonin agent, sigma receptor

Introduction

The aim of this study is to evaluate the mechanisms of action of the commercial drug Tenoten®, which has been used in the treatment of psychoneurotic and neurogenic disorders. The active ingredient of the drug is released-active form of antibodies to S100 protein (RAF of Abs to S100). Released-activity is defined as follows: pretreated ultrahigh dilutions cause modifying effect on the original substance.¹ It has been shown that drugs of this class have a fundamentally novel pro-antigenic action (codirectional with antigen), since the released-active form of the antibody alters the interaction of the specific antigen (molecule) with its target by mechanism of conformation modification.² The efficacy and safety of the drugs were intensively studied and proved in different experimental models and in clinical studies as well.³–¹¹
RAF of Abs to S100 exert effects similar to those of the S100 protein. RAF of Abs to S100 possesses membrotonic effects: induced membrane depolarization, reduced the amplitude or completely blocked the action potential, accelerated the maximal rise of the action potential, reduced maximal conductance, and facilitated the steady-state inactivation of Ca\textsuperscript{2+} channels.\textsuperscript{12} These effects are unidirectional with a regulatory effect of the S100 protein on the electrical activity of the neuron membrane.\textsuperscript{13} RAF of Abs to S100 could modify S100 protein activity.\textsuperscript{14} Moreover, RAF of Abs to S100 without exerting direct influence on the induction of long-term post-tetanic potentiation prevents the blocking action of large doses of antibodies to S100 on long-term post-tetanic potentiation, which indicates its modifying effect.\textsuperscript{15} It has been previously shown during long-term sensitization conditioning that membrane structures may play a role in the effect of RAF of Abs to S100.\textsuperscript{16} S100 protein is a low-molecular weight protein (20 kDa), detected in the body of vertebrates, which has two calcium binding sites.\textsuperscript{17} S100 protein is involved in the regulation of such fundamental processes as generation and transmission of nerve impulses, protein phosphorylation, transcription factors, calcium homeostasis, and changes in cytoskeleton components, enzyme activity, cellular growth and differentiation, and in the development of inflammatory reaction.\textsuperscript{17} In the nervous system, S100 protein is involved in the tracking of synaptic vesicles, cellular survival, and differentiation.\textsuperscript{17–20}

Experimentally and clinically, it was shown that RAF of Abs to S100 exerts unique psychotropic activity\textsuperscript{10} including anxiolytic, antiasthenic, antiaggressive, stress-protective, antihypoxic, antiischemic, neuroprotective, and nootropic effects. When evaluating the effects of RAF of Abs to S100 on rats, it was shown that blockage and stimulation of \( \gamma \)-aminobutyric acid (GABA) and serotoninergic receptors affected anxiolytic and antidepressant activity.\textsuperscript{21–23} The results from an in vivo study of anxiolytic and antidepressant activity of RAF of Abs to S100 have shown that preliminary ketanserin blockage of 5-HT\textsubscript{2A} serotonin receptors caused a reduction in intensity of the effects of RAF of Abs to S100. These data have proved the involvement of the serotoninergic system in the process, namely the involvement of 5-HT\textsubscript{2A} receptors in the realization of the effects of RAF of Abs to S100.\textsuperscript{21} Vogel conflict test results showed that GABA\textsubscript{A} receptor blockage induced by bicuculline and blockage of the chloride channel upon injection of picrotoxinin significantly reduced anti-conflict effect of RAF of Abs to S100.\textsuperscript{22} Additionally, simultaneous injection of GABA\textsubscript{A} receptor agonist baclofen with RAF of Abs to S100 reduced anxiolytic effect of the latter, while simultaneous injection of GABA\textsubscript{A} receptor antagonist phaclofen on the contrary enhanced anti-conflict effect of RAF of Abs to S100 providing further support for effecting this pathway.\textsuperscript{23}

The purpose of this study was to evaluate the influence of RAF of Abs to S100 on major neurotransmitter systems which are possibly involved in its mechanism of pharmacological activity. For that purpose, we investigated the effect of RAF of Abs to S100 on dopaminergic, serotonergic, and GABAergic receptors, different sites of N-methyl-d-aspartate (NMDA) receptors, as well as \( \sigma \), and \( \sigma \) receptors using radioligand binding and/or functional activity assays.

**Methods**

All the experiments were conducted using validated methods in accordance with standard operating procedures (SOPs) used by Eurofins Cerep (Paris, France) and Euroscreen S.A. (Brussels, Belgium). All cell membranes were provided by Eurofins Cerep (formerly Cerep) and Euroscreen S.A. All samples (positive controls, negative controls, and RAF of Abs to S100) were tested in the equal amount (v/v). Distilled water served as negative control for both RAF of Abs to S100 and placebo, while solvents or assay buffers - for positive controls. The number of replicas was in the range of two to six that corresponds to the number, which was ordinarily used in the similar in vitro experiments.\textsuperscript{24–26} Results analysis was performed as described in the respective chapters of the Assay Guidance Manual.\textsuperscript{27} The concentrations of all radioligands and nonspecific ligands used in radioligand binding assays were chosen in accordance with the results of the respective preliminary saturation binding assays and historical values determined at Eurofins Cerep or Euroscreen S.A.

The data from Eurofins Cerep and Euroscreen presented in the submitted manuscript was obtained from in vitro experiments that did not require approval from an ethical committee. Biological material originated from non-human cells expressing recombinant human proteins, immortalized cell lines and rodent tissues. In the latter case, rodents were bought from registered breeders providing us health monitoring reports and housed at Eurofins Cerep before tissue collection.

The Eurofins Cerep animal housing was approved by governmental institution in September 2011 for a 5 years period.

For tissue collection, Eurofins Cerep structure adheres to the external local ethics committee for animal experimentation, registered in December 13, 2012 by the Governmental
Radioligand assays: 5-HT$_{1F}$, 5-HT$_{2B}$, 5-HT$_{2cedited}$, 5-HT$_{3}$, and sigma$_{1}$ receptors

Receptor binding assays for the human serotonin (5-hydroxytryptamine [5-HT]) receptors 5-HT$_{1A}$, 5-HT$_{2B}$, 5-HT$_{2cedited}$ and 5-HT$_{3}$ were performed using membrane preparations from CHO-K1 cells (exception for 5-HT$_{1F}$ – Chinese hamster ovary [CHO] cells were used) expressing the respective human receptor. MCF-7 or Jurkat cells were used in the assays for human recombinant or native sigma$_{1}$ receptors, respectively. Extracts of cell membranes were incubated with the appropriate radioligands in the presence or absence of RAF of Abs to S100 protein or placebo, diluted in assay buffer. Test compounds were added to the well at 50 μL (50%, v/v) for all 5-HT receptors and for recombinant sigma$_{1}$ receptor, and at 20 μL (10%, v/v) or at the following range to generate a dose–response curve in the assay on the native sigma$_{1}$ receptor: 20 μL, 10 μL, 4 μL, 2 μL, 1 μL, 0.4 μL, and 0.2 μL (placebo was tested at 20 μL or 10% [v/v] only). Protein concentration was 5 μg/well for 5-HT$_{1A}$, 7 μg/well for 5-HT$_{2B}$, 1 μg/well for 5-HT$_{2cedited}$, 2.5 μg/well for 5-HT$_{3}$, 15 μg/well for recombinant sigma$_{1}$, or 200 μg/ well for native sigma$_{1}$. The assay buffer containing 50 mM Tris (pH 7.4) and 0.1% ascorbic acid was supplied with the following: 4 mM CaCl$_{2}$ for 5-HT$_{1A}$, 4 mM CaCl$_{2}$ and 10 μg/mL saponin for 5-HT$_{2B}$, and 5 mM CaCl$_{2}$ for 5-HT$_{3}$. In case of the recombinant or native sigma$_{1}$ receptors, 50 mM Tris (pH 8.0) or 50 mM Tris–HCl (pH 7.4) was used as the assay buffer. The following radioligands were used for each receptor binding assay: $[^{3}H]$-LSD (7 nM) for 5-HT$_{1A}$, $[^{3}H]$-LSD (1.6 nM) for 5-HT$_{2B}$, $[^{3}H]$-mesulergin (1.85 nM) for 5-HT$_{2cedited}$, $[^{3}H]$-BRL43694 (2.5 nM) for 5-HT$_{3}$, and $[^{3}H]$ (+)-pentazocine for sigma$_{1}$ receptors (61 nM or 8 nM for recombinant or native, respectively). Non specific binding was determined in the presence of a saturation concentration of the corresponding nonspecific ligand: BRL54443 (1 μM) for 5-HT$_{1A}$, ritanserin (1 μM) for 5-HT$_{2B}$, 5-HT (100 μM) for 5-HT$_{2cedited}$, MDL72220 (100 μM) for 5-HT$_{3}$, and haloperidol for sigma$_{1}$ receptors (5 μM or 10 μM for recombinant or native, respectively). All binding assays except those for recombinant sigma$_{1}$ receptor were performed by a filtration method using Filtermate Harvester (PerkinElmer Inc., Waltham, MA, USA). Recombinant sigma$_{1}$ binding assay was carried out using Filtermat A, Wallac. Filters were preliminary soaked in 0.5% polyethylenimine (PEI) for 5-HT receptors or in 0.1% and 0.3% PEI for recombinant or native sigma$_{1}$ receptors, respectively.

For the rest of the receptors tested (for the human dopamine receptors D$_{1}$, D$_{2A}$, D$_{2B}$, D$_{3}$, D$_{5}$; for the rat GABA receptors GABA$_{A}$ and the human GABA$_{B_{1b}}$ receptors; for the rat glutamate receptors pentanexyl pipеридине [PCP], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA], kainate, NMDA; for the human 5-HT receptors 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{3}$; and for the rat sigma$_{2}$ receptors), Supplementary materials list the respective experimental conditions for the binding assays.

For radioligand binding experiments, the half maximal inhibitory concentration (IC$_{50}$) and the half maximal effective concentration (EC$_{50}$) values were determined (via computer software) by nonlinear regression analysis of the competition curves using Hill equation curve fitting. The inhibition constants ($K_i$) were calculated using the Cheng–Prusoff equation ($K_i = IC_{50}(1 + (L/K_d))$, where $L$ is the concentration of radioligand in the assay, and $K_d$ is the affinity of the radioligand for the receptor.

The results are expressed as a percent of control specific binding ([measured specific binding/control specific binding] ×100) and as a percent inhibition of control specific binding (100−[(measured specific binding/control specific binding) ×100]) obtained in the presence of the test compounds.

Functional assays: $[^{35}S]$GTP$_{y}$S binding assays on D$_{3}$, GABA$_{B_{1/A/B2}}$, 5-HT$_{1A}$, and 5-HT$_{1B}$ receptors

Extracts of recombinant cell membranes of CHO expressing the respective human receptor were thawed on ice and diluted in assay buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4), 100 mM NaCl, 10 μg/mL saponin, and 3 mM MgCl$_{2}$ supplied with 0.1% bovine serum albumin for D$_{3}$ and 5-HT$_{1A}$ receptors (for 5-HT$_{1B}$, the following assay buffers were used: Krebs–Ringer HEPES, 1 mM 3-isobutyl-1-methylxanthine). They were kept on ice until assays were begun. Membranes were mixed with guanosine diphosphate (GDP) (v/v) diluted in assay buffer to reach the final concentration and incubated for at least 15 minutes on ice. In parallel, $[^{35}S]$GTP$_{y}$ (PerkinElmer, NEG030X) was diluted in assay buffer to reach a final concentration of 0.1 nM and was mixed with granules (PVT-WGA, Amersham, RPNQ0001) (v/v) directly prior to initiation of reaction.
The following compounds were successively added to the wells of an Optiplate (PerkinElmer) in order to determine agonistic activity: 50 μL of RAF of Abs to S100, or 50 μL placebo, or 50 μL of reference ligand (reference agonist – dopamine HCl for D3, SKF97541 for GABA<sub>B1A/B2</sub> and 5-CT for 5-HT<sub>1A</sub> and for 5-HT<sub>1B</sub> – diluted in assay buffer in order to reach concentration from 0.01× EC<sub>50</sub> to 100× EC<sub>50</sub>), 20 μL of membrane mixture with GDP, and 20 μL of mixture of [35S]GTPγ with beads.

The following compounds were successively added to the wells of an Optiplate (PerkinElmer) in order to determine antagonistic activity: 50 μL of RAF of Abs to S100, or 50 μL placebo, or 50 μL of reference ligand (reference agonist – GR103691 for D3, CGP54626 for GABA<sub>B1A/B2</sub> (S)-WAY100135 for 5-HT<sub>1A</sub>, and methiothepin for 5-HT<sub>1B</sub> – diluted in assay buffer in order to reach concentration from 0.01× IC<sub>50</sub> to 100× IC<sub>50</sub>), 10 μL of assay buffer, and 20 μL of membrane mixture with GDP. After a 15-minute incubation, 10 μL of reference agonist (dopamine [3 nM] for D3 and SKF97541 [8 μM] for GABA<sub>B1A/B2</sub>) in concentration equal to historical EC<sub>50</sub> and 20 μL of mixture of [35S]GTPγ with beads were added.

The plates were covered with a top seal, shaken on an orbital shaker for 2 minutes, and then incubated for 1 hour (for 5-HT<sub>1A</sub> and GABA<sub>B1A/B2</sub> – 30-minute incubation) at 21°C. Then, the plates were centrifuged for 10 minutes at 2,000 rpm, incubated at 21°C for 1 hour for D<sub>3</sub> or for 10 minutes for 5-HT<sub>1B</sub> (no incubation for 5-HT<sub>1A</sub> and GABA<sub>B1A/B2</sub>), and counted for 1 minute well with a PerkinElmer TopCount™ reader.

For the rest of the receptors tested (for the human dopamine receptors D<sub>1</sub>, D<sub>2L</sub>, D<sub>5</sub>, D<sub>3</sub>, D<sub>4</sub>, D<sub>5</sub>; for the human 5-HT receptors 5-HT<sub>1D</sub> [rat], 5-HT<sub>1E</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>2C</sub> edited, 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>6</sub>, 5-HT<sub>7</sub>), Supplementary materials list the respective experimental conditions for the functional assays.

For functional experiments, IC<sub>50</sub> and EC<sub>50</sub> values were determined (via computer software) by nonlinear regression analysis of the competition curves using Hill equation curve fitting. For the antagonists, the apparent dissociation constants (K<sub>B</sub>) were calculated using the modified Cheng–Prusoff equation (K<sub>B</sub> = IC<sub>50</sub>/(1+(A/EC<sub>50</sub>)), where A is the concentration of reference agonist in the assay, and EC<sub>50</sub>A is the EC<sub>50</sub> value of the reference agonist.

Agonist activities of test compounds are expressed as a percentage of the activity of the reference agonist at its EC<sub>100</sub> concentration. Antagonist activities of test compounds are expressed as a percentage of the inhibition of reference agonist activity at its EC<sub>50</sub> concentration.

**Test samples**

The sample tested in this study was the active pharmaceutical ingredient of the commercial drug Tenoten® (OOO “NPF “MATERIA MEDICA HOLDING”, Moscow, Russian Federation), which corresponds to RAF of Abs to S100. RAF of Abs to S100 was manufactured in accordance with the technology for the respective commercial drug production and supplied by OOO “NPF “MATERIA MEDICA HOLDING” (Russian Federation) as a ready-to-use water solution. Technology is described previously in the US patent 8,535,664<sup>28</sup> and meets applicable requirements of the European Pharmacopoeia (7th Edition, 2011). Briefly, RAF of Abs to S100 was manufactured by repeated consecutive dilution of the starting substance and external treatment during all technological steps. As a solvent, ethanol–water solution was used for intermediate dilutions, and purified water was used for preparation of the final dilution. All dilutions were prepared in glass vials in sterile conditions, avoiding direct intense light, and were stored at room temperature. Rabbit polyclonal antibodies to S100 protein (mixture of S100 protein isoforms used for the rabbit immunization), which had been concentrated approximately 2.5 mg/mL, were used as a starting substance (OOO “NPF “MATERIA MEDICA HOLDING”, Russian Federation). In the case of the placebo, the stock solution, which did not contain the rabbit polyclonal antibodies to S100 protein, was used as starting substance and underwent the same technological steps. The samples were coded by manufacturer and used blinded in the study. The remaining compounds and expendable materials used in the experiments were acquired.

**Results**

In all assays, the highest amount of RAF of Abs to S100, which could be added in the well in accordance with the SOPs used by Eurofins Cerep and Euroscreen S.A., was used. In the case of nonspecific effects of the test sample (eg, osmotic pressure cell bursts), the sample was added in the maximal possible amount, which did not cause the nonspecific effect. As a control, in parallel with the testing of RAF of Abs to S100 in each assay, vehicle (prepared using the similar dilution process) used for preparation of RAF of Abs to S100 was tested. In some cases (Tables 1 and 2) because of the high nonspecific effect of the vehicle, it was impossible to interpret the results obtained on RAF of Abs to S100, which were later considered as negative because of high nonspecific effect of vehicle. Only the results from the experiments which showed no nonspecific effect of the vehicle were analyzed.
Table 1 Effect of RAF of Abs to S100 on radioligands binding to receptors

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>% of control specific binding</th>
<th>Reference compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td><strong>Dopamine receptors</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt; (h)</td>
<td>103.60±12.42</td>
<td>SCH23390</td>
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<td>D&lt;sub&gt;2&lt;/sub&gt; (h)</td>
<td>99.73±14.97</td>
<td>Risperidone</td>
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<tr>
<td>D&lt;sub&gt;3&lt;/sub&gt; (h)</td>
<td>94.30±18.77</td>
<td>7-OH-DPAT</td>
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<tr>
<td>D&lt;sub&gt;4&lt;/sub&gt; (h)</td>
<td><strong>126.25±6.58</strong></td>
<td>R-(+)-OH-DPAT</td>
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<tr>
<td>D&lt;sub&gt;5&lt;/sub&gt; (h)</td>
<td>89.40±7.99</td>
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<td>D&lt;sub&gt;7&lt;/sub&gt; (h)</td>
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<td>Dopamine</td>
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<td><strong>GABA receptors</strong></td>
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<td>GABA A</td>
<td>96.00±29.13</td>
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<td>GABA B&lt;sub&gt;(1b)&lt;/sub&gt; (h)</td>
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<td><strong>Glutamate receptors</strong></td>
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<td>99.20±9.05</td>
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<td>AMPA</td>
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<td>NMDA</td>
<td>90.40±2.69</td>
<td>CGS 19755</td>
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<td><strong>Serotonin receptors</strong></td>
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<td>5-HT&lt;sub&gt;6&lt;/sub&gt; (h)</td>
<td>119.02±11.56</td>
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<td>Ketanserin</td>
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<td>5-HT&lt;sub&gt;2B&lt;/sub&gt; (h)</td>
<td><strong>131.9±11.88</strong></td>
<td>Ritanserin</td>
<td>7.78</td>
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<td>5-HT</td>
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<tr>
<td>5-HT&lt;sub&gt;5&lt;/sub&gt; (h)</td>
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<td>Mianserin</td>
<td>130.3</td>
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<td>139.93±18.17 HWE</td>
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<td><strong>Sigma receptors</strong></td>
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<td>Sigma&lt;sub&gt;1&lt;/sub&gt; (native)</td>
<td><strong>24.70±5.02</strong></td>
<td>Haloperidol</td>
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<tr>
<td>Sigma&lt;sub&gt;2&lt;/sub&gt; (recombinant)</td>
<td><strong>59.68±3.37</strong></td>
<td>Haloperidol</td>
<td>23.2</td>
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<tr>
<td>Sigma&lt;sub&gt;2&lt;/sub&gt;</td>
<td>102.00±14.0</td>
<td>Haloperidol</td>
<td>82</td>
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</table>

**Notes:** The results are expressed as % of control specific binding (mean ± SD, n=2–5). IC<sub>50</sub> value (concentration ensuring 50% inhibition of control specific binding) was characterized by means of assay of linear and non-linear regression of curve of concurrent binding by using Hill equation. Bold values highlight the values for the positive results.

**Abbreviations:** HWE, high non-specific effect of vehicle (the data is not applicable for interpretation); h, human; SCH23390, 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol; 7-OH-DPAT, 7-hydroxy-2-(di-n-propylamino)tetralin; 5-HT, 5-hydroxytryptamine; CGP54626, [S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]-4-(phosphonomethyl)piperidine-2-carboxylic acid; BRL-54443, 3-(1-methylpiperidin-4-yl)-1H-indol-5-ol; MDL72222, [(1S,5R)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl] 3,5-dichlorobenzoate; 5-CT, 5-carboxamidotryptamine; NMDA, N-methyl-d-aspartate; GABA, γ-aminobutyric acid; PCP, phencyclidine; piperidine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; IC<sub>50</sub>, half maximal inhibitory concentration; RAF of Abs to S100, released-active form of antibodies to S100 protein; SD, standard deviation.

**Radioligand assays**

RAF of Abs to S100 caused significant effect on the interaction of radioligands with seven receptors (Table 1). RAF of Abs to S100 increased radioligand binding to 5-HT<sub>1D</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2Collected</sub> and 5-HT<sub>3</sub> receptors of serotonergic system by 142.0%, 131.9%, 149.3%, and 120.7%, respectively. RAF of Abs to S100 enhanced radioligand binding to D<sub>1</sub> receptors by 126.3% as well. The sample also inhibited specific binding of ([3,4-H]-cyclohexylmethyl)phosphinic acid ([3H]-CGP54626) to GABA<sub>B1A/B2</sub> receptors by 25.8%. RAF of Abs to S100 significantly inhibited specific binding of radioligand to both native and recombinant human sigma receptors, but the intensity of the drug effect was different. Thus, when analyzing the effect on ligand interaction with recombinant receptor, the effect of RAF of Abs to S100 was 40.32%, while with native receptor, the effect was 75.3% (Table 1). Testing of RAF of Abs to S100 in several concentrations made it possible to reveal the drug “dose–response” effect, which was manifested in different intensity of displacement of high-affinity ligand from its binding sites.
on native receptors (Figure 1). At the same time, placebo did not cause any effect on the ligand–receptor interaction.

**Discussion**

The purpose of the current study was to determine the potential effects of RAF of Abs to S100 on the receptors of the major neurotransmitter systems which are possibly involved in its mechanism of pharmacological activity. The results provide direct evidence of drug influence on the serotonin, GABA, dopamine, and sigma receptors. These molecular interactions could explain the pharmaceutical action of RAF of Abs to S100 shown in previous experimental and clinical studies. With a wide range of pharmacological activities, together with the diversity of its clinical effects, it is difficult to understand the mechanism of its action. However, the deep interconnections between the various neurotransmitter systems, as well as the multireceptor nature of the mechanism of drug action, could be the basis of its diverse effects.
In vitro data also clarify the molecular mechanisms of antidepressant and anxiolytic effects of RAF of Abs to S100. Activation of 5-HT$_{1A}$ receptors by agonists is associated with anxiolytic and antidepressant activity. Inhibition of 5-HT$_{1B}$ receptors by antagonists could be used for treatment of anxiety and depression. Stimulation of 5-HT$_{2B}$ receptors is reported to induce a serotonin-selective reuptake inhibitor-like response in behavioral and neurogenic assays as well as to possess anxiolytic effect in the Vogel conflict test in rats. 5-HT$_{2C}$ receptors modulate serotonin neurotransmission during stress, mediate anxiety in humans and animals, and are involved both in anxiolytic and anxiogenic effects of serotonin-selective reuptake inhibitors. The stress-induced increase in serotonin turnover and release can be inhibited by 5-HT$_{2C}$ receptor activation.

The anxiolytic effect of RAF of Abs to S100 is also mediated by GABA$_A$ receptors. Earlier in vivo studies demonstrated that blocking of GABA$_A$ receptor by bicuculline or its clilor channel by pyrrotoxin significantly reduced anti-conflict effect of RAF of Abs to S100 in the Vogel conflict test. Additionally, according to unpublished data from an ex vivo study in mice, RAF of Abs to S100 significantly increased the number of benzodiazepine receptors expressed on the cellular membrane. In vitro evaluation of the drug effect on functional status of GABA$_A$ receptor was not conducted because of a lack of an appropriate method at the time of the initiation of the study.

Unlike benzodiazepine anxiolytics, RAF of Abs to S100 is also able to influence GABA$_B$ receptors in vivo and possesses properties of a GABA$_{B1A,B2}$ receptor antagonist as well as a demonstrated ability to inhibit specific binding of radioligand with this receptor in the present study.

GABA$_B$ receptors are involved in the regulation of different central nervous system functions including anxiety, memory, and nociception. It is known that GABA$_B$ receptor antagonists possess antidepressant activity and are able to improve learning in rats.

In vitro methods used in the present study revealed that RAF of Abs to S100 exerts its activity in binding assays only where RAF of Abs to S100 stimulated radioligand binding with the D$_3$ receptors, whose ligands are known to have potential as antipsychotic and antiparkinson agents. Even though the received effect of RAF of Abs to S100 could be explained by an allosteric-like action, it should be demonstrated in additional experiments taking into account that the sample did not have any effect on functional status of the dopamine receptors used in the current study.

Figure 1 Specific binding of [H]pentazocine with sigma, receptors (native) in the presence of different doses of RAF of Abs to S100.

Notes: The results are expressed as % of specific binding (mean ± standard deviation, n=6, mean data of three experiments).

Abbreviation: RAF of Abs to S100, released-active form of antibodies to S100 protein.
Taking into consideration the anti-cataleptic effect of RAF of Abs to S100, its effect on sigma receptors ensuring neuronal plasticity was analyzed. RAF of Abs to S100 reduced the number of radioligands specifically bound to the sigma receptors in a dose-dependent manner; however, it did not affect ligand–receptor interaction between sigma receptors and their radioligand. Additionally, according to unpublished data of an in vivo study, RAF of Abs to S100 under single dosing caused significant reduction in cataleptic activity of haloperidol, a ligand of the sigma receptor, on the Morpurgo’s model of catalepsy.

There are data proving that sigma receptors exert a modifying effect on all major neurotransmitter systems, including noradrenergic, serotoninergic, dopaminergic, and cholinergic system, and NMDA-regulated glutamate effects. Sigma receptors play an important role in the pathophysiology of neurodegenerative diseases (eg, Alzheimer disease, Parkinson disease), mental and affective disorders, and biological insult and are involved in the process of learning and memory. In this regard, the ability of RAF of Abs to S100 to affect the ligands’ interaction with sigma receptors might serve as one of the primary mechanisms for the realization of its pharmacological activity.

The key for understanding the possible mechanism of action on the receptor level is to demonstrate that S100 protein is the target for the RAF of Abs to S100. Thus, it was shown that RAF of Abs to S100 has similar effects as the S100 protein; direct action on membrane stabilization could modify S100 protein activity, and its effect has tight connections with membrane structures. Based on these observed effects, it could be assumed that RAF of Abs to S100 alters receptor binding and signaling through regulation of S100 cellular activities. S100 proteins regulate a diverse array of cellular activities, including membrane–cytoskeleton interactions, cytoskeleton dynamics, and membrane rearrangement, which could affect the organization of lipid domains (rafts), and effect the modulation of the receptors function. The plasma membrane is a dynamic entity. It consists of a heterogeneous but regulated environment and includes discrete membrane domains that form through a combination of lipid and protein interactions. Highly ordered structures within the membrane that are rich in saturated lipids and scaffolding proteins are termed lipid rafts. Lipid rafts along with adaptor proteins regulate monoamine receptors, in particular serotonin and dopamine receptors. It was shown that lipid rafts can alter the conformation of the G-protein-coupled receptors, in particular acetylcholine and serotonin receptors, and thus can regulate neurotransmitter binding and signal transducing functions. In addition, lipid rafts also promote the function of GABA receptors – lipid rafts affect cellular processes such as activity-dependent desensitization and receptor trafficking. Finally, the sigma receptor is enriched with lipid raft markers, and their functions are tightly connected. Directly, S100 as an adaptor protein has been found to interact with serotonin and dopamine receptors, causing an increased localization of the 5-HT and 5-HT receptors to the plasma membrane, modulates serotonin neurotransmission, and enhances D receptor signaling. Based on the current work, the exact mechanism should continue to be explored.

Conclusion

The received results provide with the first insight on the mechanism of the drug action on the receptor level. The pharmacological profiling of RAF of Abs to S100 and 27 receptor targets provides the direct evidence of a drug-related modification of the major neurotransmitter systems (serotoninergic, GABAergic, dopaminergic, and sigma receptors as well). Our results indicate that the effect of RAF of Abs to S100 on serotoninergic and dopaminergic receptors might have an allosteric nature which should be confirmed with further studies. Finding opportunities for optimization of in vitro techniques to overcome the limitations associated with the testing of water solutions will allow extending the understanding of mechanism of the drug action. Also, the demonstration of “in vitro–in vivo” correlation through further experiments including in vitro, in vivo, and ex vivo approaches should be done in order to confirm the connection between preclinical and clinical effects, which had been shown previously, with the receptor targets revealed within current work.

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Disclosure

Evgeniy A Gorbunov, Irina A Ertuzun, Evgeniya V Kachaeva, and Sergey A Tarasov are employees of the company, and Oleg I Epstein owns the company. The authors report no other conflicts of interest in this work.
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