IN VITRO AND *IN SILICO* STUDIES ON ZINC INTERACTION WITH 5-HT$_7$ RECEPTORS

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INTRODUCTION

Allostery is a mechanism that regulates function of many proteins and thus controls various biological processes [1]. There are now growing number of allosteric modulators acting on various GPCRs but in the group of 5-HT receptors only a few examples were identified. Recently we showed [2] an allosteric nature of zinc dual action at 5-HT$_{1A}$ receptors and here we investigate its influence on 5-HT$_7$ receptor subtype.

MATERIALS and METHODS

Biological material Membranes were prepared from human embryonic kidney (HEK) 293 cells stably transfected with human 5-HT$_7$ receptor cDNA.

Radioligand Binding Assays A 5-HT$_7$ receptor agonist [^{3}H]-5-CT and two antagonists [^{3}H]-SB-269970 and [^{3}H]-Mesulergine were used. The incubation buffer consisted of 50 mM Tris–HCl (pH 7.7), 4 mM $MgCl_2$, 10 mM pargyline and 0.1 % ascorbic acid. Nonspecific binding was defined by the binding obtained in the presence of 10 μM 5-HT. Radioligand binding assays were performed by incubating 5 μg of protein of the membrane suspension in 96-well microtiter plates with a final volume of 200 μl, for 60 min at 37 °C under equilibrium conditions. The binding reactions were stopped by filtration through GF/B Unifilter plates using a harvester (PerkinElmer). The plate filters were then dried, and 20 μl of Ultima Gold MV (PerkinElmer) was added. Radioactivity was measured using a MicroBeta TriLux counter (PerkinElmer).

Dissociation Assays Dissociation rate kinetic assays were performed at 37°C using the same buffer conditions described for the equilibrium binding assays and 0.8 nM [^{3}H]-5-CT, 2.5 nM [^{3}H]-SB-269970 and 10 nM [^{3}H]-Mesulergine. Non-specific binding was defined by the addition of 10 μM serotonin. Membranes were incubated with radioligand for 60 min to achieve equilibrium. Next, serotonin at fixed concentration (10 μM) or serotonin with 500 μM of $ZnCl_2$, was added. The specifically bound radioligand was measured after incubations of different durations (from 0 to 60 min), which were terminated by rapid filtration.

Functional evaluation The functional properties of agonist 5-CT (without and in the presence of 10 μM and 100 μM of Zn^{2+}) in a HEK293 cells overexpressing 5-HT$_7$R were evaluated, as its ability to increase cAMP production. I the case of antagonists, their inhibition of cAMP production evoked by 10 nM 5-CT (a concentration producing 90% (EC_{90}) of the maximum agonist activation) was assessed. The standard assay procedure from LANCE Ultra cAMP: Assay Development Guidelines [3] was used. Time-resolved fluorescence resonance energy transfer (TR-FRET) was detected by an Infinite M1000 Pro (Tecan, Männedorf, Switzerland).

Analysis of data Analysis of the saturation binding data obtained for the agonist and antagonists using the program GraphPad PRISM, according to equation [4]: $pK_{DApp}=-\log([A]+10^{logK_A})+\log(\alpha[A]+10^{logK_A})-\log d$, where log*d* is a fitting constant; K_A denotes the affinity of zinc for the allosteric site; [A] the Zn^{2+} concentration; α defines the cooperativity factor.

Molecular Dynamics The MD simulations were performed on homology models of 5-HT$_7$R, created on the basis of crystal structure of 5-HT$_{1B}$R (pdb: 4IAR). Structures of reference agonist (5-CT) and antagonist (SB-269970) were docked into the model (Glide 5.5) and the ligand-receptor complexes were input for the MD with zinc ions. Simulations sytems were constructed with POPC membrane and TIP3P water model.

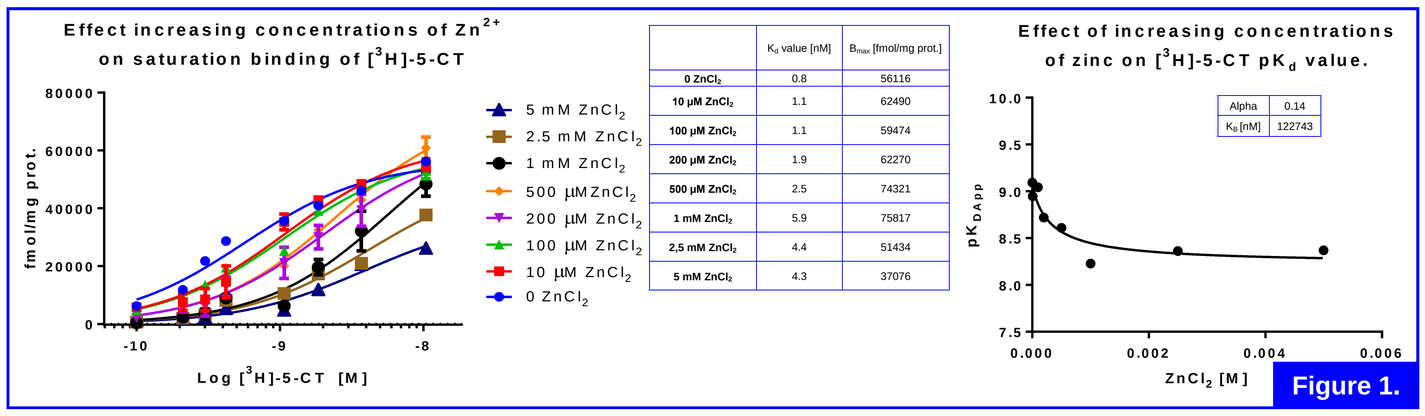
RESULTS

Analysis of saturation isotherms obtained for three radioligands (an agonist [^{3}H]-5-CT and antagonists [^{3}H]-SB-269970 and [^{3}H]-Mesulergine) and seven increasing concentrations of zinc (10 μM - 5 mM) **[Fig. 1–3]** revealed a decrease in radioligand binding (increased K_d values in relation to binding without zinc). In each case, the calculated cooperativity factor (α < 1) indicated negative allosteric modulation.

In kinetic experiments, interaction of Zn^{2+} with the 5-HT$_7$ receptor were evaluated in the absence and presence of 500 μM Zn^{2+} [**Fig. 4**]. The increase of the dissociation kinetic rate in the presence of 500 μM Zn^{2+} was observed for [^{3}H]-5-CT and [^{3}H]-Mesulergine, whereas in the case of [^{3}H]-SB-269970 no significant changes were detected.

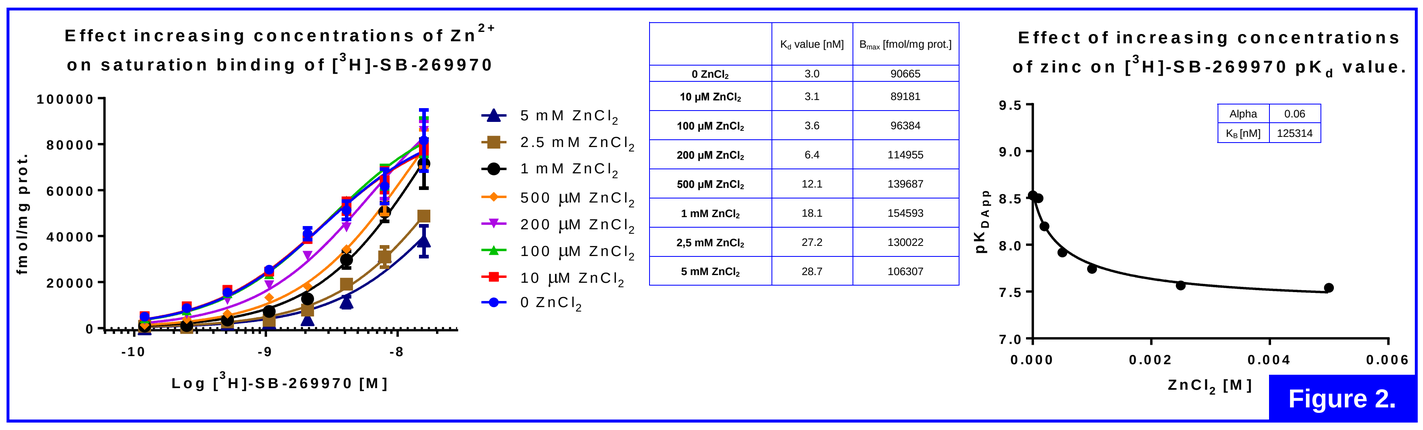
In functional cAMP assays, zinc at 10 μM and 100 μM concentrations produced rightward shift of the agonist (5-CT) dose-response curve and an increase of EC_{50} values [**Fig. 5**]. The zinc also negatively influenced on action of both antagonists inhibiting cAMP level produced by 10 nM 5-CT, however the effect was less pronounced than for agonist. **Figure 6** shows results of control experiments, i.e. the screening of different ions on radioligand binding to 5-HT$_7$ receptor. The specific action of zinc, compared to other ions, is clearly visible. **Figure 7** shows the differences in interactions with zinc ions between antagonist (left) and agonist (right) bound models of 5-HT$_7$. In both experiments zinc was contacting with ecl2 (D167 and D168), however in complex with antagonist zinc ion is also observed interacting with D3.32.

Summing up, both *in vitro* and *in silico* studies indicated that Zn^{2+} ions act as negative allosteric modulator at 5-HT$_7$ receptors.



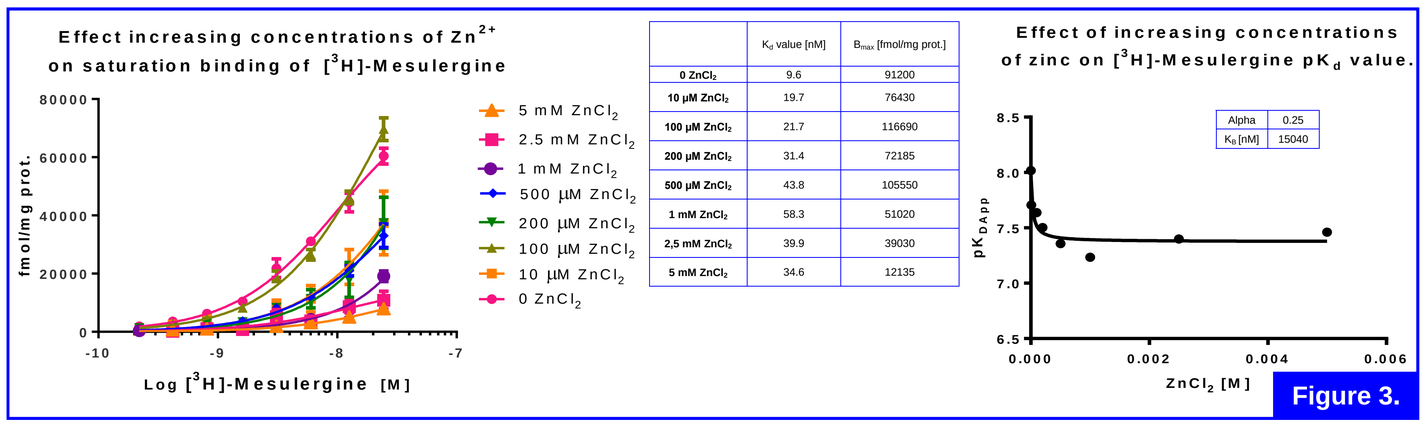
	K_d value [nM]	B_{max} [fmol/mg prot.]
0 $ZnCl_2$	0.8	56116
10 μM $ZnCl_2$	1.1	62490
100 μM $ZnCl_2$	1.1	59474
200 μM $ZnCl_2$	1.9	62270
500 μM $ZnCl_2$	2.5	74321
1 mM $ZnCl_2$	5.9	75817
2,5 mM $ZnCl_2$	4.4	51434
5 mM $ZnCl_2$	4.3	37076

Figure 1.



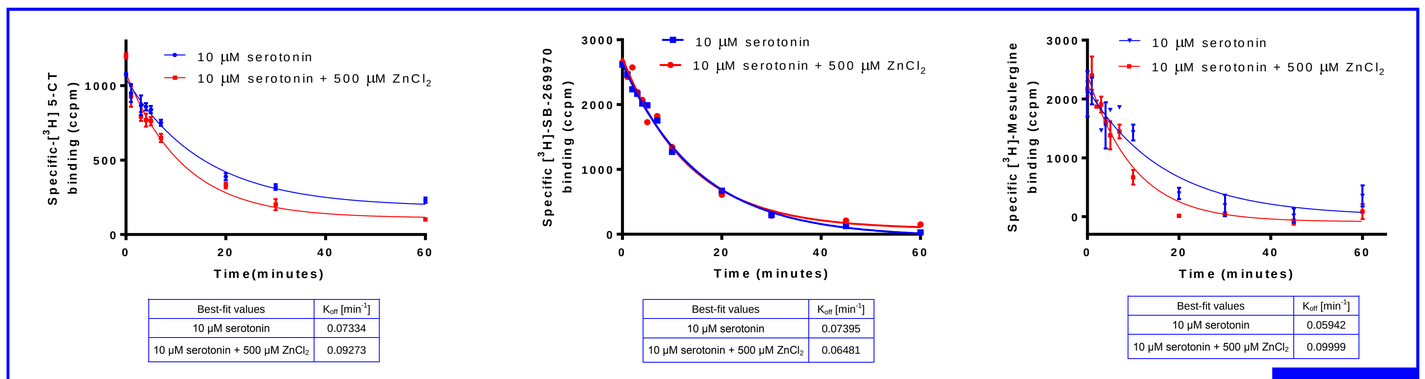
	K_d value [nM]	B_{max} [fmol/mg prot.]
0 $ZnCl_2$	3.0	90665
10 μM $ZnCl_2$	3.1	89181
100 μM $ZnCl_2$	3.6	96384
200 μM $ZnCl_2$	6.4	114955
500 μM $ZnCl_2$	12.1	139687
1 mM $ZnCl_2$	18.1	154593
2,5 mM $ZnCl_2$	27.2	130022
5 mM $ZnCl_2$	28.7	106307

Figure 2.



	K_d value [nM]	B_{max} [fmol/mg prot.]
0 $ZnCl_2$	9.6	91200
10 μM $ZnCl_2$	19.7	76430
100 μM $ZnCl_2$	21.7	116690
200 μM $ZnCl_2$	31.4	72185
500 μM $ZnCl_2$	43.8	105550
1 mM $ZnCl_2$	58.3	51020
2,5 mM $ZnCl_2$	39.9	39030
5 mM $ZnCl_2$	34.6	12135

Figure 3.

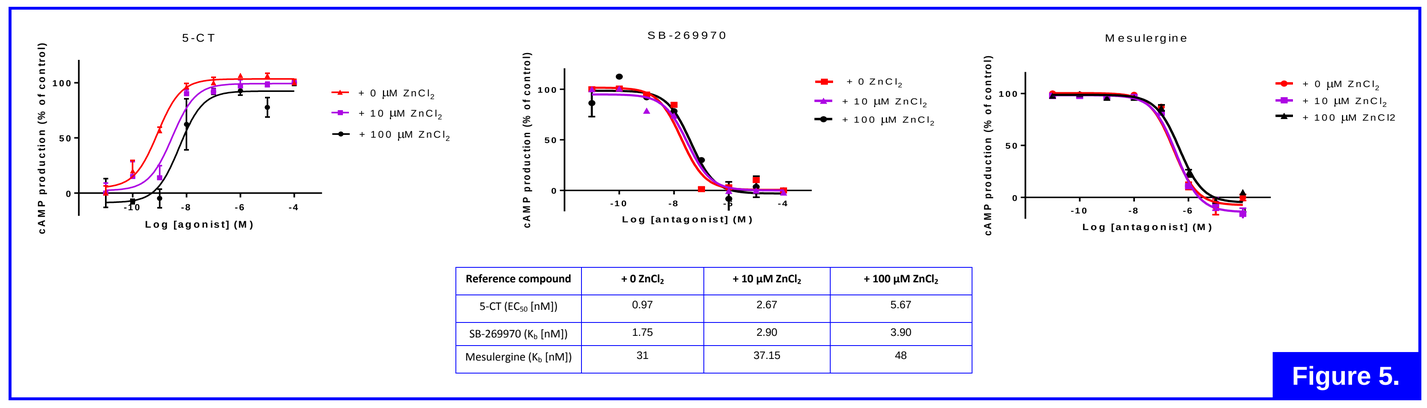


Best-fit values	K_{off} [min^{-1}]
10 μM serotonin	0.07334
10 μM serotonin + 500 μM $ZnCl_2$	0.09273

Best-fit values	K_{off} [min^{-1}]
10 μM serotonin	0.07395
10 μM serotonin + 500 μM $ZnCl_2$	0.06481

Best-fit values	K_{off} [min^{-1}]
10 μM serotonin	0.05942
10 μM serotonin + 500 μM $ZnCl_2$	0.09999

Figure 4.



Reference compound	+ 0 $ZnCl_2$	+ 10 μM $ZnCl_2$	+ 100 μM $ZnCl_2$
5-CT (EC_{50} [nM])	0.97	2.67	5.67
SB-269970 (K_b [nM])	1.75	2.90	3.90
Mesulergine (K_b [nM])	31	37.15	48

Figure 5.

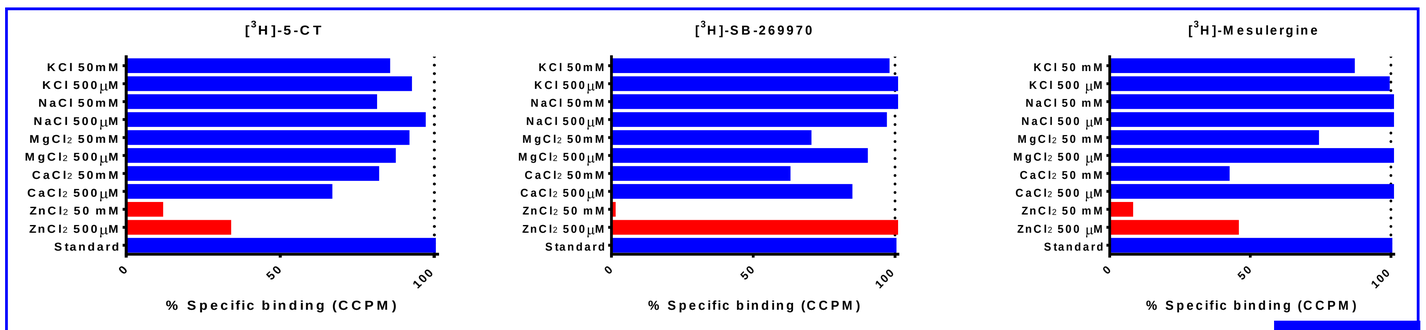


Figure 6.

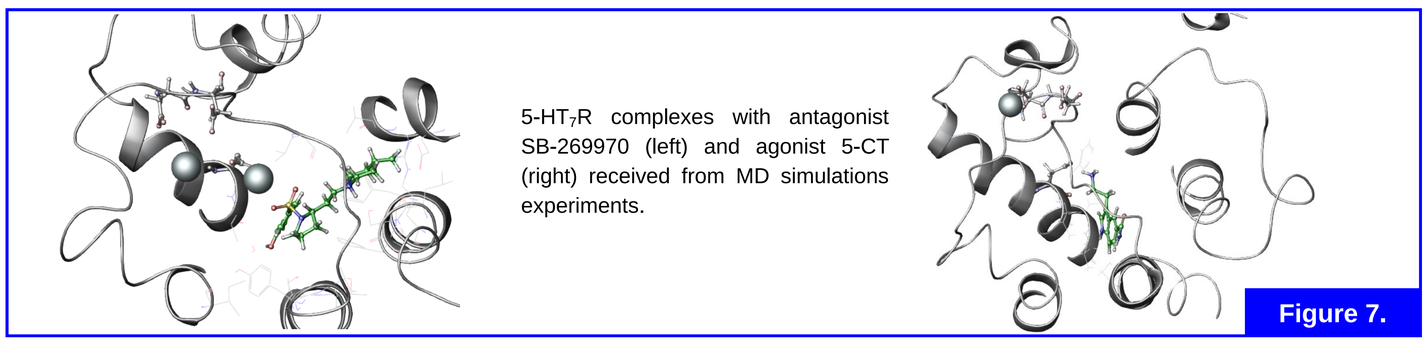


Figure 7.

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1) Christopoulos A., Kenakin T., 2002. G protein-coupled receptor allosterism and complexing. Pharmacol. Rev. 54, 323–374.
2) Satała G., Duszyńska B., Stachowicz K., Rafalo A., Pochwat B., Luckhart C., Albert P.R., Daigle M., Tanaka K.F., Hen R., Lenda T., Nowak G, Bojarski A.J., Szewczyk B.,2015, Concentration-Dependent Dual Mode of Zn Action at Serotonin 5-HT1A Receptors: In Vitro and In Vivo Studies.Mol Neurobiol. 2015 Dec 12
3) May L.T., Leach K., Sexton P.M., Christopoulos A., 2007. Allosteric modulation of G protein-coupled receptors. Annu. Rev. Pharmacol. Toxicol. 47, 1–51
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