Application of Drug Repositioning Strategy to TOFISOPAM

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Abstract: Drug repositioning strategy is an interesting approach for pharmaceutical companies ; especially to increase their productivity. SELNERGY[tm] is a reverse docking based-program able to virtually screen thousands of compounds on more than 2000 3D biological targets. This program was successfully applied to tofisopam and revealed that the isomers of tofisopam are able to fit with phosphodiesterase 4. This old drug was used as a racemic mixture to treat anxiety in the eighties and was recently shown to act as a PDE4 inhibitor. Thanks to this strategy we demonstrated that tofisopam acts *via* the inhibition of PDE4 in the submicromolar range. Moreover, we firstly showed that the S-enantiomer of tofisopam is ten times more active than R-enantiomer. The identification of the biochemical mechanism of tofisopam isomers now allows to reposition this drug in new therapeutic indications where modulation of cAMP *via* PDE4 inhibitors are possible.

Keywords: Reverse docking, PDE4, SELNERGY, chiral purification.

INTRODUCTION

It is well-known that drug discovery and development is a time consuming process. One of the bottlenecks resides in *in vivo* assay phases where important pharmacological parameters must be evaluated such as absorption (for oral medication), bioavailability, metabolism, pharmacokinetics, efficiency and toxicity.

A drug is generally used against a particular symptom but it always possesses side biological activities (e.g. side effects) which depend on how we use it and at what doses. An optimisation of side activities is proposed to find new drugs [1]. Drug repositioning or repurposing is aimed at finding new applications for a drug. Here "new applications" means applications not yet referenced for that particular drug [2, 3]. The benefits of such approach is that some pharmacological data are already available so saving time and money in the development phases. So it is a way to increase productivity of R&D.

But how to conduct a drug repositioning study? One can consider an extensive biological profiling, though this solution seems to be expensive. In order to avoid unnecessary *in vitro* tests, we propose to employ inverse docking for target selection prior to *in vitro* binding evaluation, so that experimental efforts will only be focused on the most probable protein candidates.

Here, we report our drug repositioning strategy based on our in-house tool SELNERGY[tm] applied to tofisopam [4]. This drug was selected because it is a suitable candidate for drug repositioning for three major reasons: (i) the drug is now free of patents, (ii) this is a racemic mixture of two candidates and (iii) the biochemical action of tofisopam remains unclear. Thus, many biological perspectives are possible.

Tofisopam is an atypical 2,3-benzodiazepine Fig. (**1A**) which doesn't bind to benzodiazepine receptors and devoid of CNS side-effects characteristic of 1,4-benzodiazepines. The molecule has one chiral centre. Tofisopam was marketed as Grandaxin by Egis-Servier in the racemate form and it was orally administered at 300 mg/day for its anxiolytic properties. Lastly, this racemate was shown to inhibit PDE4 and its structure was used as scaffold to conceive new potent PDE inhibitors [5].

Although *(R)*-isomers of tofisopam are actually in phase II clinical trial for irritable Bowel Syndrome, the biological target remains unknown. The goal of our drug repositioning strategy is to identify new biological targets for existing drug and use this drug in new therapeutic area in which this new identified biological target is engaged.

1. MATERIALS AND METHODS

1.1. Computational Chemistry: *(R)*- and *(S)*-Tofisopam Structure Calculation and SELNERGY

The two enantiomers were sketched in Sybyl6.9 (Tripos Inc., St. Louis, MO, USA) and their 3D structure calculated by Concord (Pearlman, Tripos Inc., St. Louis, MO, USA) as implemented in Sybyl6.9. Energy minimisation was subsequently applied to the structures. The minimisation procedure was described elsewhere [6].

SELNERGY[tm] consists of a database of protein targets from crystallography or homology modelling. The target structures are annotated according to their source (e.g. human, bacterial,...), their protein family and their therapeutic applications [6]. Currently, this library contains 2000 structures. The docking engine of SELNERGY[tm] is based on FlexX 2.0 [7]. We ran FlexX with the ChemScore set of parameters. A statistical method called Multiple Active Site Corrections (MASC) [8] was also applied to score the ligand/protein pairs.

2.2. Analytical Chemistry: HPLC Separation of Tofisopam Conformers

Compound and Reagents

Tofisopam racemate mixture was purchased from Sigma-Aldrich (St. Louis, MO, USA) under the reference T8200.

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Rolipram was purchased from Sigma-Aldrich (St. Louis, MO, USA) under the reference R6520. Heptane and ethanol (HPLC grade) were purchased from J.T. Baker (Noisy le Sec, France).

Apparatus

The liquid chromatographic apparatus consisted in a Varian Prostar pump Model 230 Solvent Delivery Module (Varian, Les Ulis, France), a Rheodyne (Berkeley, CA, USA) model 7125 injector with a 200 µL sample loop, a Varian Prostar Model 330 Photodiode Array (PDA) detector and a Jasco Circular Dichroism Chiral Detector Model CD-2095 (Jasco International Co., Maryland, USA). The UV data from PDA ranging from 200 to 350 nm were collected by a Star Chromatography Workstation (Varian, Les Ulis, France). Data handling and chromatogram generation from CD detector have been performed using EZStart Elite software, version 3.1.4 (Scientific Software, Pleasanton, CA, USA). The selected wavelength with CD detector was 230 nm.

The chiral separation was carried out on a silica-based cellulose tris-methylbenzoate Chiralcel OJ-H column (250 x 4.6 mm ID) packed with 5µm particle size, preceded by a Chiralcel OJ-H (10 x 4 mm ID) pre-column (Chiral Technologies Europe, Illkirch, France). Separation was performed using a normal phase methodology with a mobile phase consisting of n-Heptane/Ethanol (90:10, v/v) was eluted in isocratic mode with at 1 $mL.min^{-1}$.

The column was placed in a block type heater Gecko-Cil oven (Cil Cluzeau, Sainte-Foy-La-Grande, France). The system was left 2 h to equilibrate before injecting the first sample. In order to avoid a radial temperature gradient in the column, the mobile phase was pre-heated by means of a stainless steel tube (3 m x 0.25 mm ID) in the column heater after the injector. The optimised temperature was 30°C.

Calculations

The selectivity factor (α) was calculated as k_2/k_1 and retention factors (k) as $k_1 = (t_1–t_0)/t_0$ and $k_2 = (t_2–t_0)/t_0$ where t_1, t_2 refer to the retention times of the first and second enantiomers, respectively. The resolution factor (R_S) was calculated by the formula:

$$R_S = 2\,(t_2–t_1)/\,(\omega_1 + \omega_2)$$

where ω_1 and ω_2 are the peak widths for the first and second eluting enantiomer peaks, respectively. The peak of the solvent front was considered to be equal to the dead time (t_0) and was taken from each particular run. It was about 3.2 min. Retention times were mean values of two replicate determinations.

2.3. Biological Assays: Phosphodiesterase (PDE) Activity Tests

PDE1, PDE3, PDE4 and PDE5 were isolated by anion exchange chromatography from bovine aortic smooth muscle cytosolic fraction according to [9] and stored until use at -80°C in small aliquots (100 µL). PDE2 was isolated from human platelets and stored until use at -80°C in small aliquots.

PDE activities were measured by radio-enzymatic assay as previously described [10] at a substrate concentration of 1µM cAMP or 1 µM cGMP in the presence of 10,000 cpm [3H]-cAMP or [3H]-cGMP as tracers. The buffer solution was of the following composition: 50 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, 50 mg/L BSA. PDE1 was assayed at 1 µM cGMP in calmodulin-activated state (18 nM calmodulin with 10 µM $CaCl_2$). PDE2 was evaluated at 1 µM cAMP + 1 mM EGTA in cGMP-activated state (in presence of 5 µM cGMP). PDE3 and PDE4 were assayed at 1 µM cAMP + 1 mM EGTA. To prevent the influence of reciprocal cross-contamination between PDE3 and PDE4, the studies were always carried out in the presence of 50 µM rolipram for PDE3 and in the presence of 50 µM cGMP for PDE4. PDE5 activities were measured at 1 µM cGMP in the presence of 1 mM of EGTA.

Compounds were dissolved in DMSO then diluted in water so that the maximum final concentration in DMSO is 1%. At this concentration, DMSO did not affect PDE activity.

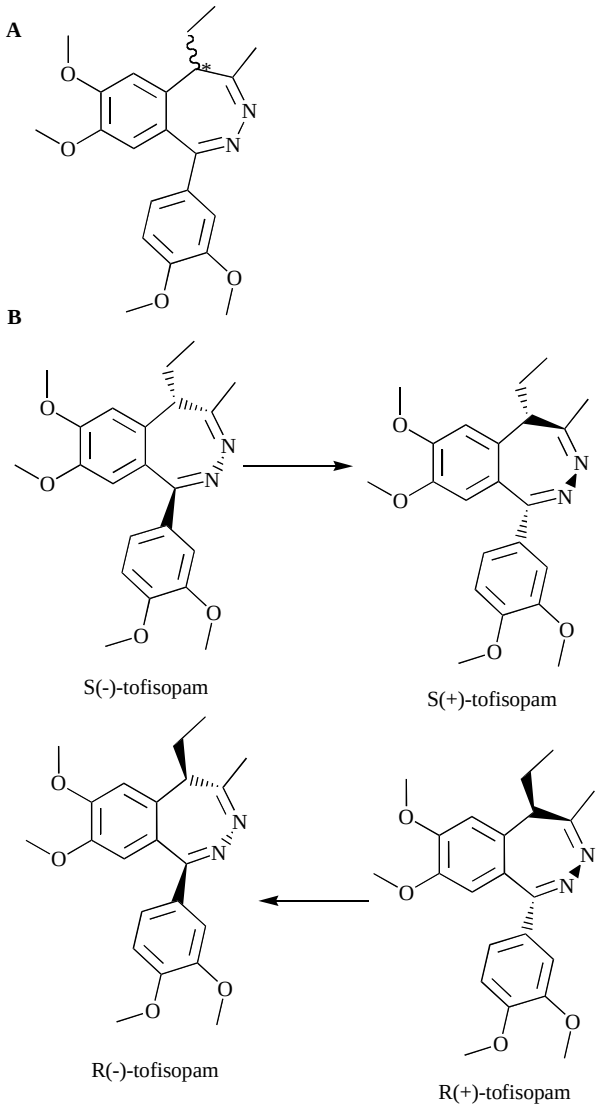

Fig. (1). The structure of the racemate tofisopam (**A**). *(S)*- and *(R)*-enantiomers of tofisopam and their possible conformers. Conformational interconversion of tofisopam enantiomers where the thick arrows show the more stable conformer and so the major conformers in solution (**B**).

The concentration of compounds that produced 50% inhibition of substrate hydrolysis (IC_{50}) was calculated by non linear regression analysis (GraphPad Prism software, San Diego, Ca) from concentration-response curves and included 6 different concentrations of inhibitors. The results represent the mean value of 3 determinations with experimental error around 15%.

3. RESULTS AND DISCUSSION

3.1. Molecular Modelling: Inverse Docking Results from SELNERGY

The 3D calculated structures of two enantiomers of tofisopam showed different ring "flip" depending on the stereochemistry of the chiral centre Fig. (**1B**) resulting in four possible forms R-(+), R-(-), S-(+) and S-(-). Only two of them have been proposed by Concord. Interestingly, we observed interconvertions between the two conformers of each isomer, with two major forms, R-(+) and S-(-), as can be seen in the purification section.

We ran Selnergy with the two major conformers. It resulted in a list of putative targets for *(S)*- and *(R)*-isomers (Table **1**). Targets selected by Selnergy are ranked in increasing MASC energy. The best protein partner of the *(S)* form seems to be a staphylococcal nuclease, followed by phosphodiesterase 4 (PDE4), isopenicillin N-synthase and uroporphyrinogen decarboxylase. *(R)*-enantiomer may bind to squalene-hopene cyclase, protein-tyrosine phosphatase, PDE4, ferredoxin $NADP^+$ reductase. Phospholipase A2 was discarded after visual inspection whereas staphylococcal nuclease and squalene-hopene cyclase were well positioned in the active sites.

PDE4 is a common target for *(R)* and *(S)* and is an interesting target involved in numerous therapeutic domains [11]. That is the reason why we decided to investigate this enzyme further. Examining the co-crystallised structures of *(R)*- and *(S)*-rolipram, the two enantiomers are positioned in the same fashion in the PDE4 active site as seen in the PDB structure 1OYN. The predicted activity of *(R)*-rolipram is 2.5 folds better than *(S)*-rolipram one. The placements of *(R)*- and *(S)*-tofisopam inside PDE4 were also similar Fig. (**2**). The MASC score is better for the *(R)*/PDE4 pair than for the *(S)*/PDE4 pair. In order to analyse *in vitro* the PDE4 inhibiting activities of the tofisopam enantiomers, we undertake the separation and the purification of the two forms of tofisopam.

3.2. Purification of *(R)*- and *(S)*-Tofisopam

Tofisopam was synthesised and sold in the form of the racemic mixture containing four different forms as shown in Fig. (**1B**). However, the overall separation of the four isomers, one pair of enantiomers and another pair of conformers, evidently needs the application of chiral separation. Two only studies having made a success of separation of four conformers were noted in literature. The first assigned four peaks analysing the ethanolic solution of tofisopam by affinity chromatography on human serum albumin stationary phase [12]. The second discloses another resolution of tofisopam using Chiralcel OJ (Daicel) as a stationary phase and n-hexane, 2-propanol and methanol (72:1.5:3, v/v/v) as a mobile phase, in a method taking more than 40 minutes to elute the enantiomers [13].

An analytic method employing any chromatographic technique was reported by Tóth *et al.* [14]. The racemic mixture with their corresponding *(R)*-conformers may be converted to the *(S)*-dibenzoyltartaric acid salt, which is a diastereomeric mixture of configurations. The pair of diastereomers possesses different properties, e.g., differential solubility, that allows for the use of conventional separation methods. This resolution has been successfully applied to the resolution of racemic tofisopam. But, solvents used were highly toxic like chloroform and the complete purification required a second step which was chiral analytical column [14].

It was found that most of the experimental separations were unsatisfactory because the chiral separation media were either incapable of providing satisfactory resolution of tofisopam enantiomers, or demonstrated long retention times when optimised to resolve the tofisopam enantiomers. So, it needed to work out a more elaborated method in order to isolate enantiomers with high purity.

Table 1. List of Putative Targets from SELNERGY[tm]

Molecules	PDB id	Protein name	MASC[d]
(R)-tofisopam	1stb	Staphylococcal nuclease[b]	0.472
	1oyn	Phosphodiesterase 4D2 (PDE4D2)[c]	0.661
	1hb3	*Isopenicillin N-synthase*	1.004
	1r3t	*Uroporphyrinogen decarboxylase*	1.130
(S)-tofisopam	1kqu	*Phospholipase A2*[a]	1.239
	1sqc	Squalene-hopene cyclase[b]	1.239
	1bzc	*Protein-tyrosine phosphatase*	1.626
	1oyn	Phosphodiesterase 4D2 (PDE4D2)[c]	1.821
	1baf	Ferredoxin $NADP^+$ reductase	1.834

[a]represent pairs discarded after visual inspections; [b]visually convincing solutions; [c]predictions validated experimentally; [d]MASC = Multiple Active Site Correction.

The chiral separation results were presented in Table **2**. In Table **2**, data are the retention factors (k), selectivity factors (α) and resolutions (Rs) of the tofisopam conformers in a normal phase separation on CHIRALCEL OJ. Because of the good solubility of tofisopam in n-hexane and ethanol, the k values of most conformers did not change much with the change of mobile phase composition. To simultaneously determine the four conformers, a mobile phase of n-hexane/ethanol (90:10, v/v) was selected as a compromise between the required resolution and speed of analysis. Fig. (**3**) shows a chromatogram of a standard solution spiked at a level of 0.2 $mg.L^{-1}$ using the above mobile phase. As it can be seen, four chromatographic peaks were obtained for tofisopam. Moreover, CHIRALCEL OJ-H allowed the separation of the enantiomers of tofisopam with a resolution higher than 2.5, in ethanol with n-hexane as mobile phase. A column filled with 5µm particle size stationary phase and n-hexane/ethanol mixtures as mobile phase was used rather than 10µm particle size and n-hexane/isopropanol mixtures [14] to obtain higher resolution values.

The UV spectra are shown in Fig. (**3**) for the four eluted peaks: as expected, the UV absorbance of the separate conformers were identical with maximum wavelengths at 205.2, 232.5, 308.0 nm. That's why a wavelength of 230 nm was chosen for detecting compounds with Circular Dichroism detector. Low wavelength allows a higher sensitivity, because this detector was less sensitive than UV detector. The UV wavelength was 310 nm in order to avoid saturating the signal during injection of a large volume of tofisopam solution.

Fig. (**4**) illustrates the results obtained for the separations of racemic tofisopam on the Chiralcel OJ-H column with UV and CD detections. The identification of the enantiomers peaks could be directly performed by intensity of UV response and polarity of CD signal, and the data of previous

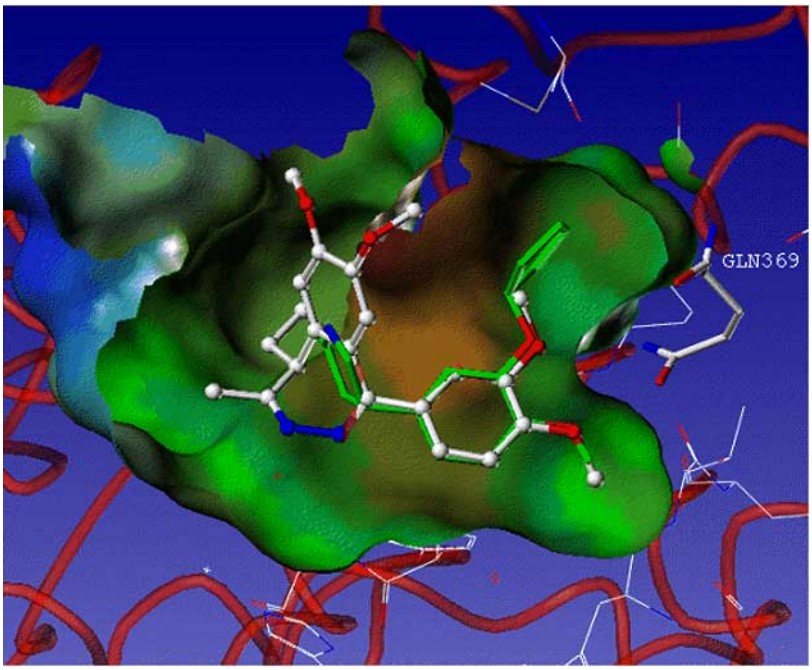

Fig. (2). *(R)*-tofisopam (carbon atom in white, in ball and stick representation) as docked in PDE4 and comparison with the co-crystallised rolipram (carbon atom in green, in capped stick display). The red tube corresponds to the protein backbone. The active site is mapped with a surface coloured by hydrophobic properties. Hydrophilic zones are in blue, hydrophobic ones in brown and intermediate in green.

Table 2. HPLC RESOLUTION of Tofisopam Isomers on Chiralcel OJ-H Column

Compounds	t (min)[a]	k[b]	α[c]	Rs[d]
R-(-)-tofisopam	13.69	3.28		
			1.21	3.67
S-(+)-tofisopam	15.94	3.98		
			1.13	2.69
S-(-)-tofisopam	17.65	4.52		
			1.14	2.6
R-(+)-tofisopam	19.73	5.16		

[a]t = retention times; [b]k = retention factors; [c]α = selectivity factor; [d]Rs = resolution.

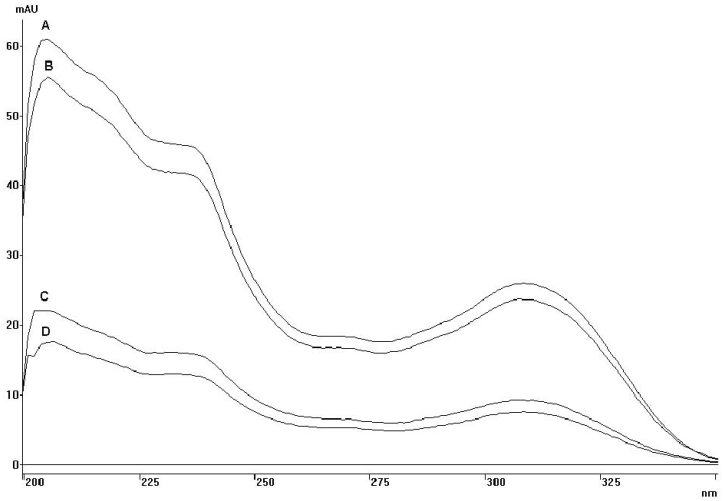

Fig. (3). UV spectra of the tofisopam isomers. Plots A, B, C and D are the spectra of S-(-)-, R-(+)-, R-(-)- and S-(+)-tofisopam, respectively.

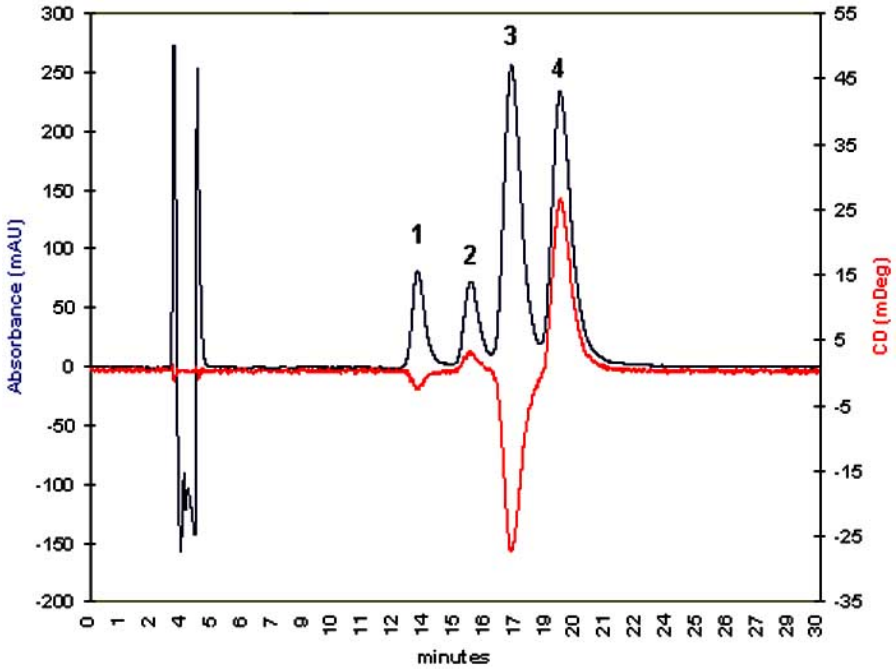

Fig. (4). Enantioseparation of tofisopam on Chiralcel OJ-H. Mobile phase = hexane/ethanol (90:10, v/v); flow-rate= 1.0 mL/min; temperature: 30°C. The blue plot represents UV signal at 310 nm. The red line represents circular dichroism response at 230 nm. Elution order is R-(-)-tofisopam (1), S-(+)-tofisopam (2), S-(-)-tofisopam (3) and R-(+)-tofisopam (4).

investigations [14]. Because Zsila *et al*. demonstrated that the (+)-conformers generate the formation of a positive Cotton effect curve, and generate a positive signal in CD detector, while the (-)-conformers generate the formation of a negative Cotton effect curve, and generate a negative signal in CD detector [14].

In accordance with the published scheme of conformer equilibrium of energetically preferred conformers Fig. (**1B**) [15] the major peaks in UV represent the isomers S-(-) and R-(+) followed by the minor peaks of the two unpreferred conformers R-(-) and S-(+)-tofisopam. The R-(-) isomer was the first compound to elute from the column, followed by S-(+), then the major isomer S-(-) and the other more represented R-(+) isomer.

These results demonstrate an equilibrium in solution between the R-(+) and R-(-) conformers and the S-(-) and S-(+) conformers. The equilibrium composition of *(R)*-tofisopam and *(S)*-tofisopam in solution is about 80% of the R-(+)-conformer and about 20% of the R-(-) conformer (Table **3**). Same results were noted for *(S)*-enantiomer. The equilibration continues during chromatographic separation [16]. The major R-enantiomer conformer, R-(+), is retained most by the separation medium, and the R-(-) conformer is the least retained. Just to the opposite, the major S-enantiomer conformer, S-(-) is more retained than the minor *(S)*-enantiomer, S-(+). Throughout the separation, the major conformers equilibrate at the finite rate to the minor conformers. This results in broadening of the bands and may serve to significantly lower yields of enantiomers isolated *via* chroma-

tographic resolutions. Thus, separation conditions that achieve resolution of tofisopam enantiomers in a shorter time interval are advantageous because the equilibration of the conformers of *(R)*- and *(S)*-tofisopam as a function of time is minimised, and the yield of the major conformer of *(R)*- and *(S)*-tofisopam is increased. It was the reason why the run time of one separation should be as short as possible for keeping elevated resolutions to collect peak after column and to recover enantiomers with high purity.

The R-(+) and S-(-) conformers were collected, dried and stored for use in biological assays. Approximately 18% of the R-(+)-tofisopam in fraction is converted into R-(-)-tofisopam if left in solution for 24 hours, corresponding in results showed in Table **3**. The same ratio resulted between S-(-) and S-(+). A 82/18 stable equilibrium R-(+)/R-(-) and S-(-)/S-(+) were observed between the two pairs of conformers in n-Heptane/ethanol (90:10, v/v) solution at room temperature.

After multiple repetitive injections, an amount of several mg of enantiomers was purified and used for biological tests. In order to calculate the enantiomeric purity, the different fractions were injected on the same chromatographic system and the chromatograms were showed in Fig. (**5**).

The *(R)*-enantiomer of tofisopam, substantially free of the *(S)*-enantiomer of tofisopam, is isolated in an enantiomeric purity of greater than 96% enantiomeric excess. The *(S)*-enantiomer of tofisopam, substantially free of the R-enantiomer of tofisopam, is isolated in an enantiomeric purity of greater than 96.6% enantiomeric excess, as determined by analytical chromatography.

3.3. Biological ACTIVITIES: PDE Profiles of the *(R)*- and *(S)*-Tofisopam

A selectivity profile of phosphodiesterase subtypes was realised and results are presented in (Table **4**). The data demonstrated an inhibition activity of tofisopam on PDE4 in a submicromolar range for the racemate and *(S)*-tofisopam, with some selectivity (5 to 40 fold according to the various tested PDEs). Interestingly, the *(S)*-isomer is ten times more active on PDE4 than the *(R)*-isomer. The *(R)* form is less active on the five PDE subtypes and poorly selective (from 0.6 to 6 fold toward PDE2, PDE3 and PDE1) in comparison with *(S)*-tofisopam. Therefore it should be noticed that the IC_{50} of *(R)*-tofisopam for PDE2 is lower than the IC_{50} for PDE4, indicating that *(R)*- tofisopam is merely selective for PDE2 in opposite to *(S)*–tofisopam selective for PDE4.

Table 3. Results of Isolation of Tofisopam Enantiomers by HPLC According to UV Response at 310 nm

	Racemic mixture		R- isolation		S- isolation	
	% SC[a]	% EC[b]	% SC[a]	% EP[c]	% SC[a]	% EP[c]
R-(-)-tofisopam	9.7	51	18.2	96	1	3.4
R-(+)-tofisopam	41.3		77.8		2.4	
S-(+)-tofisopam	9.3	49	0.8	4	18.3	96.6
S-(-)-tofisopam	39.7		3.2		78.3	

[a]%SC = relative stereoisomeric content; [b]%EC = relative enantiomeric content before isolation; [c]%EP = relative enantiomeric purity after isolation.

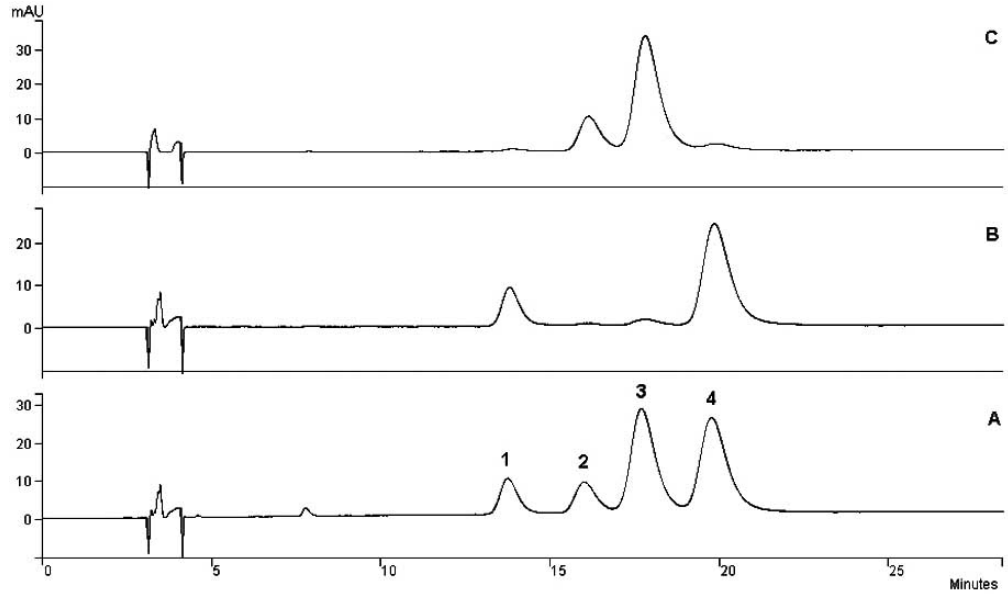

Fig. (5). Chromatograms obtained on Chiralcel OJ-H at λ = 310 nm with hexane/Ethanol (90:10, v/v) for the chiral separation of racemic tofisopam (**A**), conformers *(R)*- after isolation (**B**), and conformers *(S)*- after isolation (**C**). Temperature 30°C. Assigned peaks are R-(-)-tofisopam (1), S-(+)-tofisopam (2), S-(-)-tofisopam (3), and R-(+)-tofisopam (4).

Table 4. Selectivity Profile of Tofisopam and its Enantiomers on Five Subtypes of Phosphodiesterases

	IC_{50} (μM)				
	PDE1 (activated)	**PDE2 (activated)**	**PDE3**	**PDE4**	**PDE5**
rolipram	> 100	> 100	> 100	1.1	> 100
tofisopam (racemate)	13	3	11	0.9	36
(S)-tofisopam	13	2.3	2.8	0.6	25
(R)-tofisopam	35	3.7	17	6.1	229

SELNERGY[tm] could retrieve the "genuine" protein partner within our target library of 2000 structures. However, it failed to select the most potent conformer. A possible cause of this may be the similar placements in the PDE4 active site for the *(R)* and *(S)* forms.

CONCLUSION

In a drug repositioning strategy, with SELNERGY[tm] (Ph. BERNARD *et al*, 2004, unpublished data), a virtual biological profiling program we have confirmed and precised the effectives conformations of an old marketed drug used for central nervous system (CNS) disorders as an inhibitor of PDE4. This drug named tofisopam belongs to the pharmacological class of benzodiazepine. Interestingly, atypical 2,3 benzodiazepine nucleus of tofisopam (known by the Grandaxin name) does neither induce the classical known side-effects of benzodiazepines (sedative effect) nor bind the benzodiazepine receptor. Furthermore, *(S)*-tofisopam (IC_{50} = 0.6 μM) is two fold more active than rolipram (IC_{50}= 1.1 μM) on PDE4 in the same conditions. The main side effect of rolipram, as PDE4 inhibitor, is emesis and mild gastrointestinal distress at doses approaching 15 mg a day. The dose prescribed for this antidepressant use ranges between 0.5 to 1 mg taken three times daily. Since tofisopam was prescribed by oral administration up to 300 mg a day, and no emetic effects were recorded, *(S)*-tofisopam presents a better therapeutic index than rolipram and could be used as a PDE4 inhibitor without the major side effect: emesis. Moreover, we could develop very potent derivatives from *(S)*-tofisopam by small changes in the overall structure. Therefore, Selnergy was helpful to retrieve for old drug as well as for new compounds the "genuine" protein partner within our target library of 2000 structures and increases medicinal chemistry potentialities.

LIST OF ABBREVIATIONS

α = Selectivity factor

BSA = Bovine serum albumin

cAMP = Cyclic adenosine monophosphate

CD = circular dichroism

cGMP = Cyclic guanosine monophosphate

CNS = Central nervous system

DMSO = Dimethyl sulfoxyde

EGTA = Ethylene glycol tetraacetic acid

IC_{50} = Inhibition concentration 50%

k = Retention factor

MASC = Multiple active site corrections

NADP = Nicotinamide adenine dinucleotide phosphate basic

PDA = Photodiode array

PDB = Protein data bank

PDEi = Phosphodiesterase i

R_S = Resolution factor

t = Retention time

UV = Ultra Violet

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