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Identification of Novel Multifunctional Compounds for the Treatment of Some Aging Related Neurodegenerative Diseases

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Abstract: Aging related neurodegenerative disorders such as Parkinson disease (PD) and Alzheimer's disease (AD) are the result of multiple pathophysiological pathways that contribute to the neurodegenerative cascade. Hence, multifunctional drug candidates able to interact with several targets are of great interest for the treatment of such diseases. Therefore, an experimental and virtual screening pathway to generate multifunctional hits showing promise for the treatment of PD or AD was suggested. Moreover, suitable experimental and virtual screening methods to rapidly test pre-focused compound libraries were developed and validated. In particular, the screening was focused on potential inhibitors of acetylcholinesterase (AChE) and monoamine oxidase B (MAO B) using a combination of *in vitro* enzymatic tests, docking and scoring approaches and refined molecular modeling tools.

Keywords: Acetylcholinesterase · Experimental screening · Monoamine oxidase B · Neurodegenerative diseases · Virtual screening

Introduction

Multiple pathophysiological pathways contributing to the neurodegenerative cascade are involved in aging related disorders such as Parkinson disease (PD) and Alzheimer's disease (AD). Because of this multifaceted etiology of PD and AD, new therapeutic strategies are based on the development of multifunctional drug candidates that are able to interact with several selected targets. In fact, even if the concept of combining drugs that interact with different therapeutic targets is feasible, the development of multifunctional drugs may avoid the administration of several preparations with potentially different pharmacokinetic properties [1] and therefore diminish the risk of potential drug–drug interactions. Moreover, such multifunctional drugs interacting with different targets simultaneously may provide a greater therapeutic effect due to their synergistic actions [2]. Finally, the compliance of AD or PD patients towards a complex therapeutic regimen is low and therefore a simplification of the therapeutic regimen would be beneficial.

One of the research projects of the pharmacochemistry group at the Geneva-Lausanne School of Pharmacy (EPGL) is devoted to the identification of novel multifunctional hit compounds. First, several targets relevant for PD and/or AD were selected. Then, an experimental and virtual screening pathway for the generation of multifunctional hits showing promise for the treatment of these two disorders was suggested. Work is in progress to develop and validate suitable and complementary experimental and virtual screening methods to rapidly test pre-focused compound libraries according to the pathway suggested. In addition, set up and improvement of more complex experimental models and refined molecular modeling techniques is in progress to further characterize the most interesting hits. To develop and validate these methods, techniques and assays a series of coumarin derivatives already identified as potent inhibitors of rat brain MAO B [3] and moderate inhibitors of acetylcholinesterase [4] was selected.

Experimental and Virtual Screening Pathway to Generate Multifunctional Hits Potentially Interesting for the Treatment of PD or AD

The pathological aspects of Parkinson disease and Alzheimer's disease being quite complex, the therapeutic strategy of these two aging related neurodegenerative disorders can be focused on a large variety of potential targets such as acetylcholinesterase, monoamine oxidase B, catechol-O-methyltransferase, secretases, β-amyloid deposition, oxidative stress, neuronal apoptosis, M_2 muscarinic receptors, N-methyl-D-aspartate (NMDA) receptors, and α2-adrenergic receptors. In the search for novel multifunctional hits of interest for the treatment of PD or AD five of these

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targets were selected according to the scientific background of the pharmacochemistry group at the EPGL [3–5]. Three of them are common to both diseases, namely acetylcholinesterase, monoamine oxidase B and oxidative stress, and two targets are specific for only one of the two disorders, catechol-O-methyltransferase for PD and β-amyloid deposition for AD respectively. To identify compounds that interact at least with two of the selected targets, the screening pathway presented in Fig. 1 was suggested.

Abnormalities in cholinergic function are reported in AD and PD, but the implication of the cholinergic system in these two disorders is opposite. Due to the loss of cholinergic neurons in AD, tested compounds inhibiting acetylcholinesterase (AChE, E.C. 3.1.1.7) can be administered in the treatment of AD to enhance synaptic acetylcholine levels. In contrast, the increase of activity of cholinergic neurons being a pathological feature of Parkinson disease, AChE inhibitors have to be avoided and molecules not able to inhibit AChE are selected for subsequent screening on targets involved in PD.

Monoamine oxidase B (MAO B, E.C. 1.4.3.4) is the second target selected for the screening pathway proposed. The increase of MAO B activity being age-related, this enzyme is implicated in both AD and PD. It is now recognized that the augmentation of MAO B may cause an increase in oxidative stress, which represents one of the major factors involved in neurodegeneration [6]. Therefore, all compounds are tested for their capacity to inhibit MAO B.

Compounds devoid of AChE inhibitory activity are investigated as inhibitors of catechol-O-methyltransferase (COMT, E.C. 2.1.1.6), an enzyme involved in the pathophysiology of Parkinson disease but not of Alzheimer's disease. COMT is an important target for the treatment of PD due to its implication in the metabolism of dopamine. Co-administration of a COMT-inhibitor with levodopa prolongs the action of an individual dose of L-DOPA, currently the most effective therapy in Parkinson disease, enhancing its availability and half-life [7].

On the other hand, acetylcholinesterase inhibitors are tested for their activity to inhibit β-amyloid aggregation. Deposits of amyloid β-peptide (Aβ) in senile plaques are a characteristic hallmark of Alzheimer's disease but not of Parkinson disease. Aβ is a small peptide fragment (40–42 amino acids) of the β-amyloid precursor protein (APP). There is actually increasing evidence that Aβ is inherently neurotoxic and that it can enhance the effect of other neurotoxins [8]. Hence, compounds that prevent β-amyloid aggregation can be a great promise for treating or even preventing Alzheimer's disease.

According to the screening pathway shown in Fig. 1, all compounds are finally studied for their antioxidant properties, even if compounds inactive as inhibitors of AChE, MAO B and COMT could already be discarded at this stage because they are not potential multifunctional hits. In the complex pathogenesis of AD and PD oxidative stress plays a crucial role [9][10]. In fact, being the consequence of increased generation of reactive hydroxyl radicals, superoxide and nitric oxide, it contributes significantly to the mechanisms of irreversible cell injury associated with aging related neurodegenerative diseases.

Given the similar tasks and goals of experimental and virtual screening, the screening pathway proposed is mostly applicable to both approaches. With the exception of β-amyloid aggregation, colored in magenta in Fig. 1, all targets selected are also suitable for virtual screening. Indeed, targets colored in cyan are accessible to receptor-based *in silico* approaches and targets colored in yellow to ligand-based *in silico* techniques. β-Amyloid aggregation being a more complex process, reliable computational approaches are not yet available to correctly predict its inhibition.

Compounds that interact with only one of the selected targets are discarded because they are not multifunctional hits. On the other hand, compounds that are active at least on two of the tested targets are multifunctional hits and therefore studied in complex experimental and virtual pharmacodynamic and pharmacokinetic models to generate a lead collection.

As evidenced in Fig. 1 by coloring the complex pharmacodynamic and pharmacokinetic models in magenta, cyan and yellow, interactions of compounds with complex targets, as for example neuronal antiapoptotic properties, can not always be competently predicted by virtual approaches. Nevertheless, a large variety of refined *in silico* models can be developed either by receptor-based or ligand-based approaches.

Examples of complex virtual and experimental pharmacodynamic models relevant for the development of lead compounds of interest for the treatment of aging related neurodegenerative diseases are i) ligand-based 3D-QSAR models for MAO inhibition [3][11][12], ii) refined receptor-based virtual models of AChE and MAO B (reported below in the section 'Refined Molecular Modeling: Binding Modes on MAO B and AchE') and iii) cell-based assays to assess neuronal antiapoptotic properties of compounds. Indeed, apoptosis is considered to be a common type of neuronal cell death in AD and has been proposed to be a target for neuroprotective strategies. Hence, the potential of hits identified as dual inhibitors of AChE and MAO B to prevent apoptosis in rat pheochromocytoma differentiated PC-12 cells is currently under investigation in collaboration with the Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, University of Pisa, Italy.

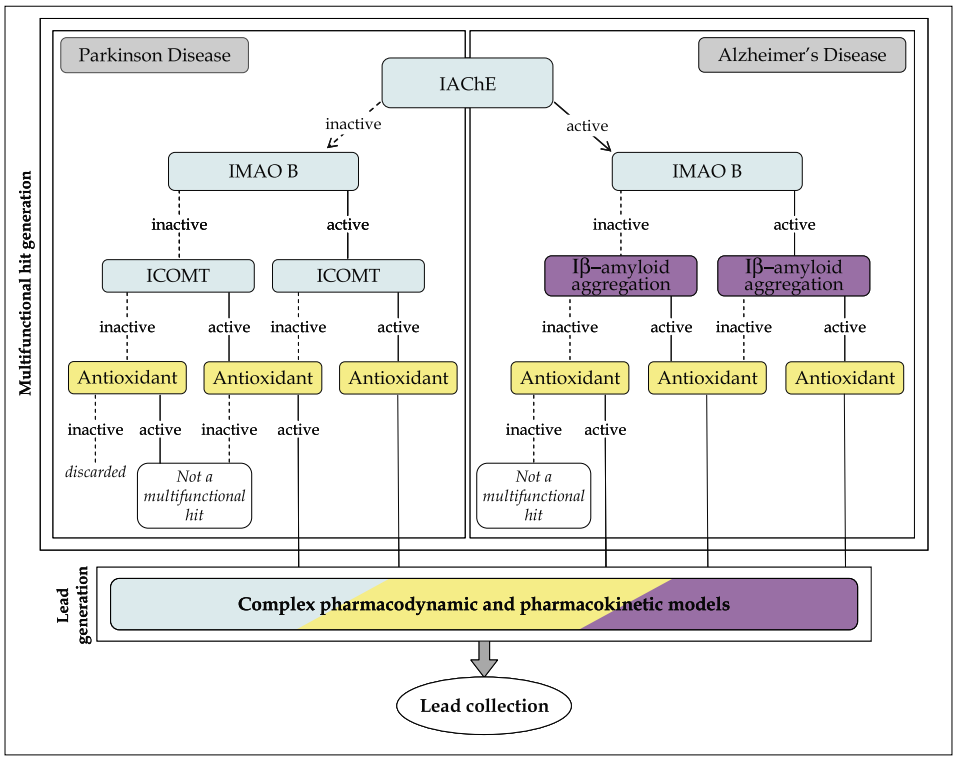

Fig. 1. Experimental and virtual screening pathway to generate hits of interest for the treatment of Parkinson and Alzheimer's disease. Cyan: targets accessible to receptor-based modeling; yellow: targets only suitable for ligand-based modeling; magenta: models currently inaccessible to molecular modeling.

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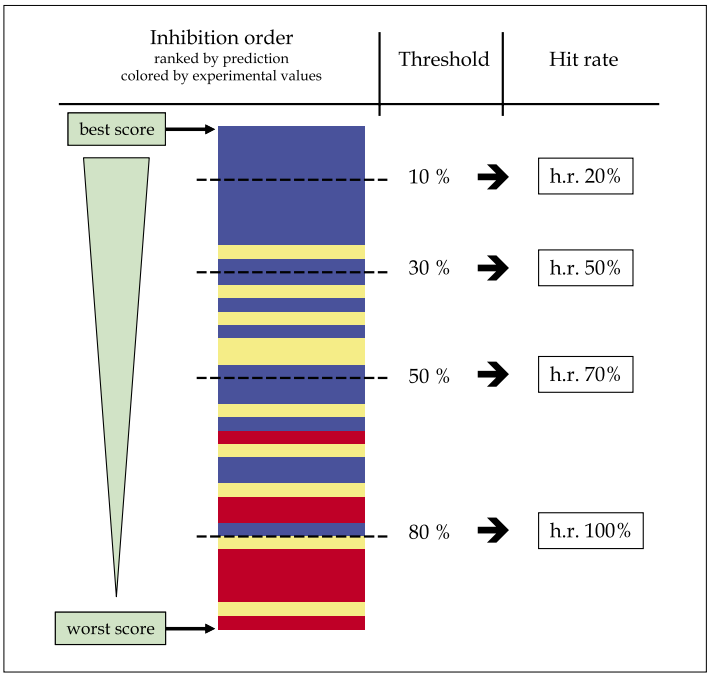

Fig. 2. 70 coumarin derivatives ranked for their *in silico* predicted inhibition activities towards MAO-B (best top, worst bottom) and colored for their *in vitro* inhibition activities: 30 are strong (blue), 24 intermediate (yellow) and 16 weak inhibitors (red). The second column is the threshold of best predicted compounds retained. The third column (hit rate) is the percentage of strong inhibitors found applying the threshold.

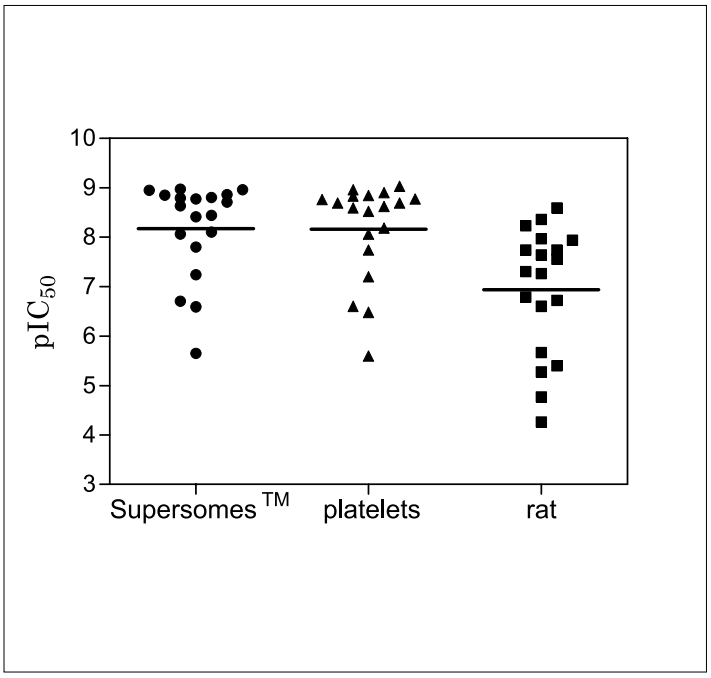

Fig. 3. Distribution of pIC_{50} values of a series of coumarin derivatives determined with human recombinant, human blood platelet and rat brain MAO B

Virtual Screening: Ranking of MAO B Inhibitors

In the last years, virtual screening tools were proposed as *in silico* filters able to rapidly identify useful hits when biostructural information on the target is available. With these approaches the pharmacochemist is able to generate a virtual focused library with an efficient flow rate [13][14]. The panel of computational methods used in virtual screening may be divided into two classes:

- Approaches allowing the generation of complexes between chemical compounds and biological targets, namely the docking methods.
- Approaches allowing the classification of the solutions obtained in the docking step in order to identify the most promising hits, namely the ranking methods.

Although virtual screening tools are nowadays extensively used, comparisons of the efficiency of theoretical and experimental screening approaches are rarely reported in the literature. Since the structure of the two targets selected (AChE and MAO B) is known, the inhibitors explored in this study were used to gain information about the convergence between virtual and experimental methods and also to refine virtual screening strategies.

Starting with the standard commercial software Gold version 2.1 [15], the docking procedure was carefully optimized by taking into account a better definition of protein cavity hydrophobicity and water molecules present in the active site.

Fig. 2 presents the comparison of MAO B inhibition for 70 coumarins as determined by virtual and experimental screening. The inhibitors are ranked according to *in silico* inhibition activities while they are colored for their experimental inhibition activities (blue for strong, yellow for intermediate and red for weak inhibitors). As displayed, a perfect prediction was not achieved, but the global separation between strong and weak inhibitors appears reasonable. However, this result underlines that the threshold used to select virtual hits has to be carefully chosen. Indeed, the risk of a too small threshold is to miss numerous experimental hits and generates a less well defined ratio between strong and intermediate inhibitors. Moreover, it must be emphasized that the threshold needed to retain all experimental hits is not compatible with the desired size reduction of chemical libraries. This example illustrates the difficulty of virtual screening strategies, namely to correctly select the balance between a sufficient reduction of a chemical database and the risk to loose some interesting hits. It also suggests that useful *in silico* procedures have to be based on successive steps by increasing thresholds.

Human Recombinant versus Rat Brain MAO B as Enzyme Source for Inhibitor Screening

To evaluate the reliability of human cloned MAO B (Supersomes™, BD Gentest) as enzyme source for the screening of MAO B inhibitors, pIC_{50} values of a series of coumarin derivatives, known as rat brain MAO B inhibitors [3], were determined for cloned human and human platelet MAO B.

As shown in Fig. 3, within experimental errors, the same inhibition potencies (pIC_{50} values) were obtained with both human MAO B sources. Indeed the correlation between the inhibitory potencies determined with human recombinant and human platelet MAO B demonstrates a linear relationship (Eqn. 1):

$$pIC_{50\ platelets} = 1.023(\pm 0.054) \cdot pIC_{50\ Supersomes^{TM}} - 0.20(\pm 0.45)$$

$$n = 19;\ r^2 = 0.99;\ s = 0.10;\ F = 1550 \qquad (1)$$

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where n is the number of compounds investigated, r^2 the squared correlation coefficient, s the standard deviation of the residuals and F the Fischer test for significance of the Eqn. 95% confidence limits are given in parentheses.

In contrast, rat MAO B inhibition potencies of all coumarin derivatives tested were lower in comparison to data obtained for the human enzyme (Fig. 3). Hence, if recombinant human MAO B was shown to be a reliable enzyme source for MAO B inhibitor screening, remarkable species-dependent differences have been pointed out by the comparison between human and rat enzyme.

Although the factors contributing to these species-dependent differences in activity remain to be identified, some hypotheses can be formulated. The comparison of the amino acid sequence for human and rat reveals considerable similarities. Rat and human MAO B have an 88% sequence identity and a 93% sequence homology [16]. However, as demonstrated by the results obtained in this work, it seems that a high degree of similarity does not necessarily imply functional identity. In fact, even if there are significant homologies in amino acid sequences between the human and rat enzymes, differences exist that may influence the inhibitor specificity of each species. As also reported by Geha *et al.*[17], it might be possible that, in spite of the high similarity in the primary amino acid sequence, there may be variations in the secondary or tertiary structure, which might explain the different specificities.

This species difference between rodent and human has crucial implications for comparative studies of drugs acting as MAO B inhibitors. Indeed, the results obtained suggest that *in vitro* MAO B inhibition values might not be quantitatively extended *a priori* from rat to human. Therefore, the enzyme source selected for the screening assay integrated in the screening pathway of hit generation for the treatment of aging related neurodegenerative disorders is human recombinant MAO B.

Development of a Screening Assay for Antioxidant Protection of Proteins

To rapidly monitor protein oxidation and antioxidant capacity to protect proteins from oxidative stress, a 96-well plate method was developed. The assay is based on a functional test using the decrease of the esterase activity of butyrylcholinesterase (BChE)-contaminated human serum albumin (HSA) as a marker of oxidative damage. The antioxidant activity of a compound is measured by its ability to preserve catalytic effectiveness despite free radical attack.

AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) was chosen as free-radical generator because it undergoes spontaneous thermal decomposition to generate two carbon-centered free radicals at a constant rate [18]. The remaining esterase activity after oxidation in presence or absence of antioxidant was calculated as percentage of the esterase activity obtained in the absence of oxidation, which was considered to be 100%. Antioxidant activity of test compounds was determined at six antioxidant concentrations and expressed as pIC_{50} values which were obtained by curve fitting according to the classical sigmoidal dose-response equation. Fig. 4 illustrates the dose-response plot obtained for ascorbic acid.

Preliminary studies allowed the optimization of the albumin concentration, organic co-solvent and oxidation conditions (temperature and AAPH concentration). Moreover, the stability of the fluorescence indicator (BCECF) and its adsorption to the microplate was investigated.

To evaluate the microtiter plate method, the antioxidant capacities (pIC_{50} values) of five model antioxidants were determined and compared to those obtained with a conventional method reported by Salvi *et al.* [5]. As shown in Fig. 5, protective activities of the antioxidants tested were in good agreement with the results reported in the literature. As the screening assay allows the rapid investigation of the antioxidant capacity of compounds to protect proteins from oxidative stress, it seems appropriate to integrate it in the screening pathway for hit finding for the treatment of Parkinson and Alzheimer's disease. Further validation of the assay with additional antioxidants is currently in progress.

Refined Molecular Modeling: Binding Modes on MAO B and AChE

The proposed screening pathway was applied to a series of synthetic coumarin

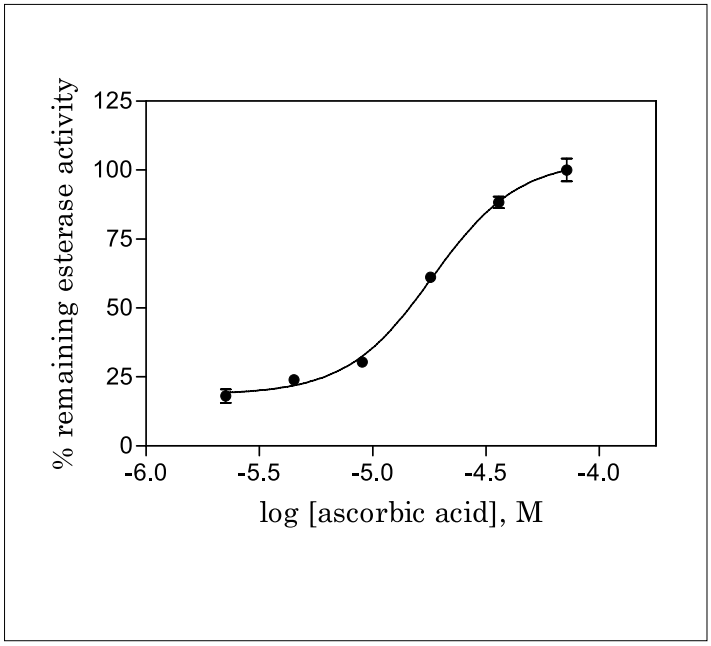

Fig. 4. Antioxidant protection by ascorbic acid of BchE-contaminated HSA against AAPH induced protein oxidation

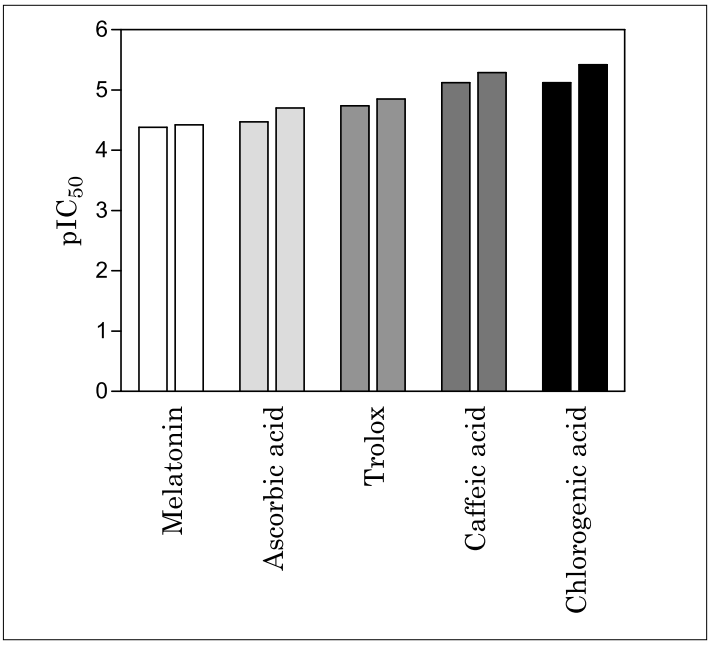

Fig. 5. Comparison between reference antioxidant activities (pIC_{50} values) determined by the developed microtiter plate method (left column) and those reported by Salvi *et al*. [5] measured by a conventional spectrofluorimetric method (right column)

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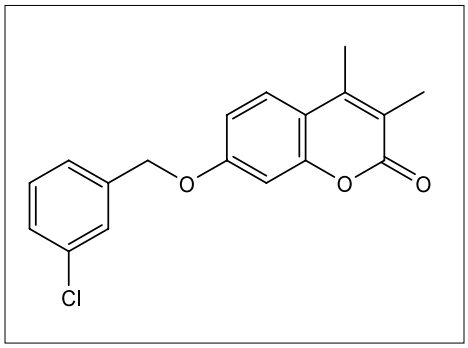

Fig. 6. Chemical structure of 7-[3-(chlorobenzyl) oxy]-3,4-dimethylcoumarin **(1)** (pIC_{50} MAO B = 8.48; pK_i AChE = 5.47).

derivatives and it allowed the identification of 7-[3-(chlorobenzyl)oxy]-3,4-dimethylcoumarin (**1**) as the most interesting multifunctional compound among this series (Fig. 6). This interesting hit has to be optimized and some structural modifications can be envisaged.

To help the design of new derivatives, it is useful to identify more precisely the binding mode of this compound to AChE and MAO B in order to identify the structural variations that could increase AChE inhibition without decreasing MAO B inhibition. The refined molecular strategy applied was the following:

- Detailed docking studies using Gold and FlexX softwares [19], in particular by assessing all the solutions obtained in different simulations.
- Structure refinement of retained solutions by force-field geometry optimization and/or molecular dynamics.
- Identification of structural families using cluster analysis.
- Ranking of retained inter- and intra-family solutions using several scoring functions (consensus scoring).
- Selection of the 'best' binding modes with the graphical display of the ScoreMLP post-processing tool based on the compatibility of polar and hydrophobic inhibitor–enzyme interactions [20].

Fig. 7 and Fig. 8 display the binding mode proposed for coumarin derivative **1**, respectively on MAO B and AChE. In both cases, the complementarity of the inhibitor and the enzyme in terms of polar and hydrophobic interactions is balanced between favorable and unfavorable regions. Some of these destabilizing lipophilic forces may be counterbalanced by specific electrostatic or hydrogen bond interactions. As a result, this first analysis suggests that improvement of this hit by structural modification is possible. It is noticeable that proposed binding modes of compound **1** display interactions with the catalytic zones of both active sites, namely the flavine-adenine dinucleotide co-factor (FAD) for MAO B and the triad (serine 200, glutamic acid 327 and histidine 440) for AChE.

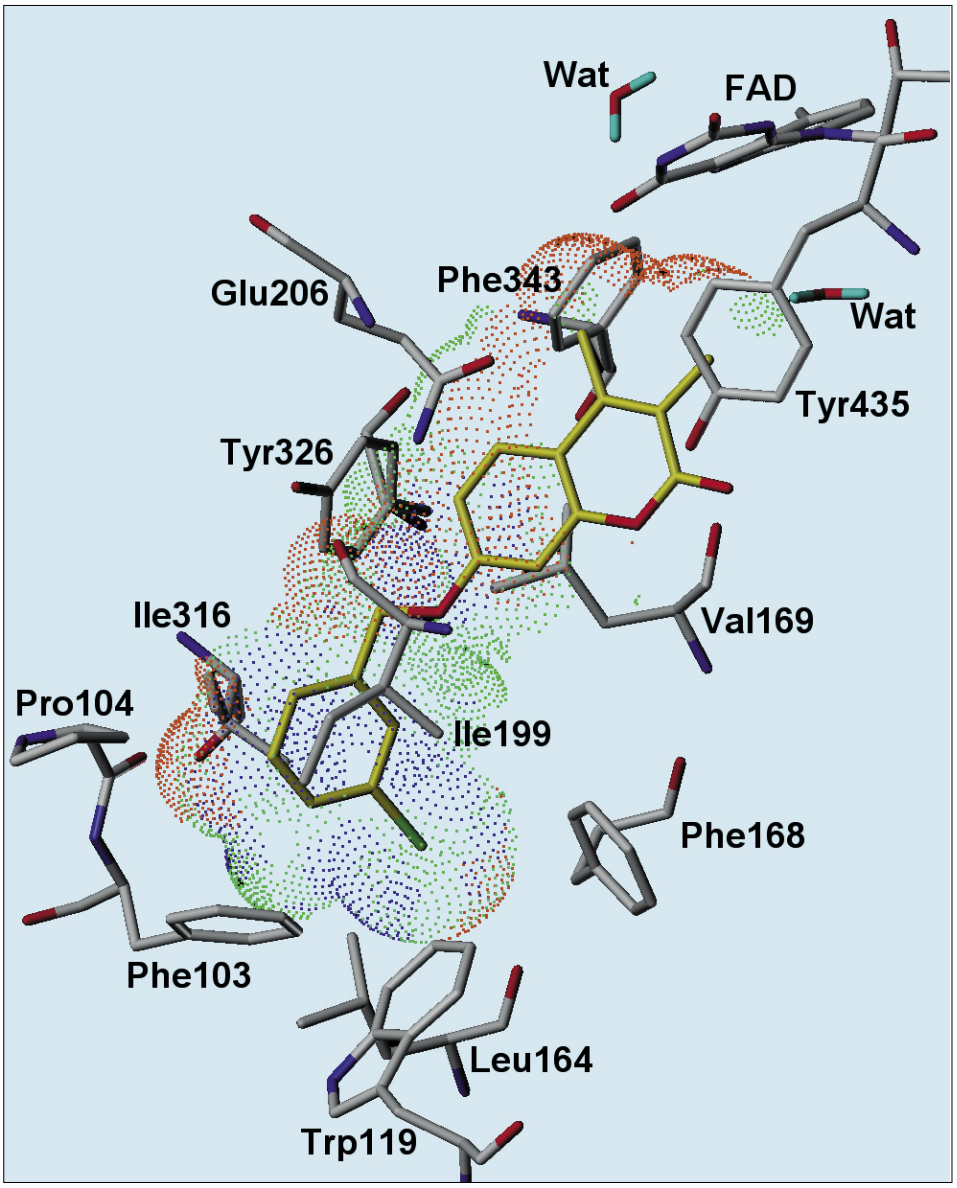

Fig. 7. Predicted binding mode of coumarin derivative **1** (carbon atoms in yellow) inside MAO B. In atom-type colors are represented the amino acids coating the binding site and potentially interacting with coumarin derivative **1**. Two water molecules (Wat) and the flavine-adenine dinucleotide co-factor (FAD) are kept during docking simulations, scoring assessment and the post-processing ligand/protein interaction analysis. The colored dots are the graphical display of the ScoreMLP post-processing tool (color coding from the most dissimilar regions to the most similar regions according to the following scale: red, orange, yellow, white, green, green-blue, blue).

Conclusion

Multifunctional compounds able to interact with several targets implicated in aging-related neurodegenerative disorders as AD and PD are a great promise for the treatment of these diseases. The experimental and virtual screening pathway proposed in this contribution allows the identification of multifunctional hits potentially interesting for the treatment of AD and PD. Moreover, it was pointed out that experimental and *in silico* screening are highly complementary disciplines in multifunctional hit generation and optimization.

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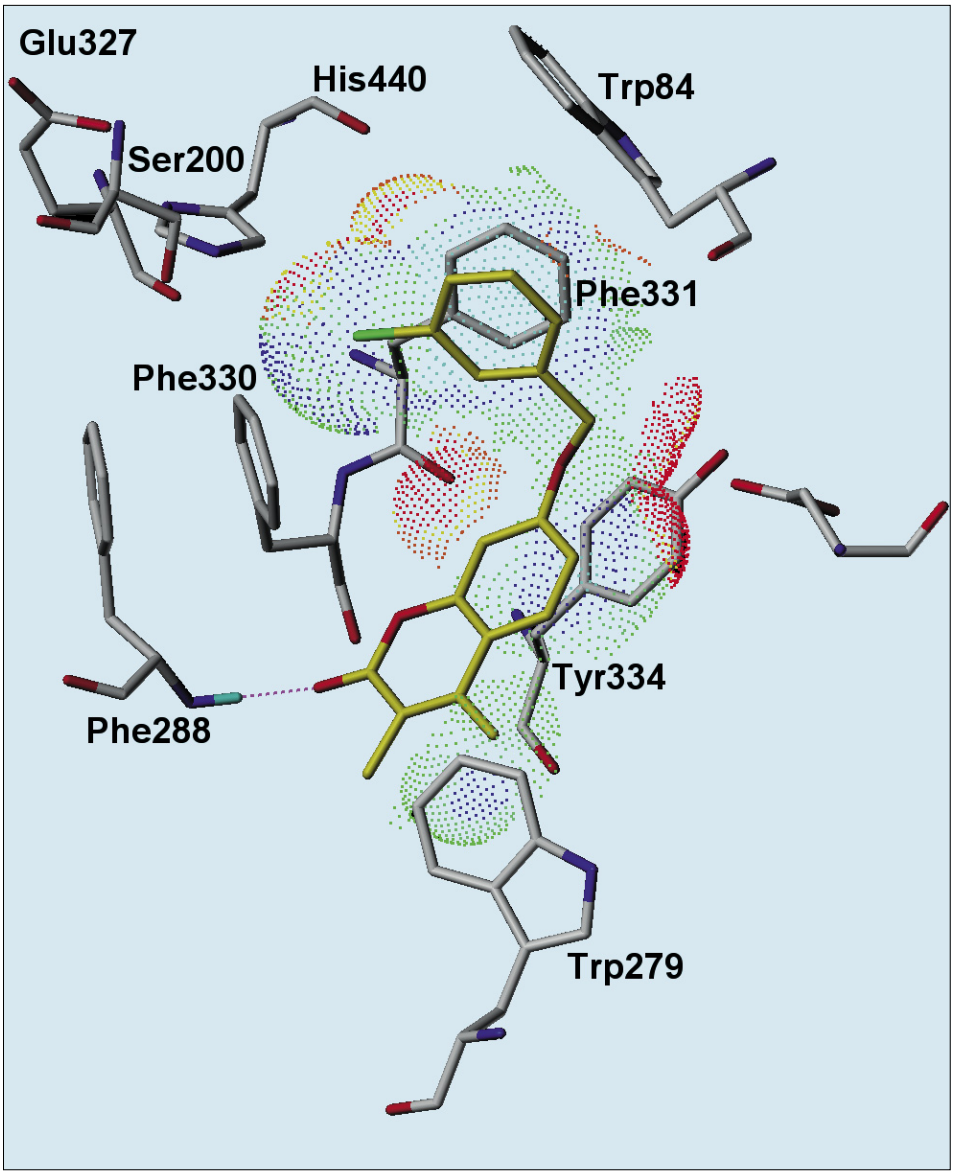

Fig. 8. Predicted binding mode of coumarin derivative **1** (carbon atoms in yellow) inside AChE. In atom-type colors are represented the amino acids coating the binding site and potentially interacting with coumarin derivative **1**. One hydrogen bond stabilizing the complex is represented in magenta (dashed line). The ScoreMLP post-processing tool is represented as a color scale on surface dots (see Fig. 7 for color coding).

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