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How to Increase the Safety and Efficacy of Compounds against Neurodegeneration? A Multifunctional Approach

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Abstract: Successful drug design requires not only the detailed knowledge of the pharmacokinetic and pharmacodynamic profiles of the drug candidate portfolio but also a thorough documentation of the possible toxic effects on humans and the environment. Thus, experimental and computational strategies able to measure or predict specific profiles of designed compounds related to their potential toxicity are highly desired. Moreover, a strategy to avoid toxic effects thus enhancing the potential efficacy of drug candidates is of great interest. To fulfil this aim, the pharmacochemistry research unit at the EPGL has recently developed and improved methodologies that detect the potential human health and environmental hazards of compounds active against neurodegeneration at an early stage. A three-step strategy is presented herein. In particular, i) an alternative index to model the bioconcentration of chemicals in the environment was determined; ii) the antioxidant activity of chemical species against free radicals was evaluated. Moreover, since antioxidants play a key role in both toxicity prevention and neuroprotection, iii) the potential interaction of such compounds with enzymatic targets involved in the neurodegenerative cascade was investigated *in silico*.

Keywords: Antioxidant activity · Bioconcentration · HDAC · Neurodegeneration

Introduction

One of the major requirements for drug development is the protection of human safety. This is especially true in the case of drugs for treating neurodegenerative disorders affecting the elder population. Different strategies should be used in order to avoid unacceptable consequences of drug candidates on both human health and the environment.

The search for new entities to fight against neurodegeneration has been widely considered through the inhibition of acetylcholinesterase and monoamine oxidase.[1,2] Such inhibition may be therapeutically useful, since it provides symptomatic treatment to diseases such as Alzheimer's and Parkinson's, respectively. Currently, new targets are being explored in order to find disease-modifying strategies, instead of merely symptomatic ones. In this way, histone deacetylases (HDACs) have been considered as potential targets against neurodegeneration.[3] Moreover, a multi-targeted drug discovery approach seems to be promising. Enzymatic inhibitors presenting antioxidant properties are of great interest in the case of neurodegeneration because of their protective role against radical species. These so-called *free radicals* can be generated not only under pathological conditions, but also from the metabolism of certain compounds that can lead to severe toxicological risks.[4,5]

The necessity to acquire information about the chronic effects of pharmaceuticals, such as the accumulation of drugs and their metabolites in the environment, is now widely recognized and required for registration of new medicinal products in the EU (directive 2006/121/EC; regulation EC no. 1907/2006). The bioconcentration factor (BCF) is normally used to quantify this property.[6] Such a parameter describes the likelihood of a chemical to concentrate in organisms when the compound is present in the environment.[7] Fish is the principal target organism used for BCF assessment due to its relevance as food for humans and to the availability of standardized testing protocols. Since the experimental determination of BCF is expensive and time-consuming, there is a need for alternative and reliable protocols capable of predicting bioconcentration of chemicals in order to estimate their potential environmental and toxicological risks.[8,9]

The research unit of pharmacochemistry has been involved in several cooperative projects to develop and apply methodologies able to rapidly identify the potential toxicity of new chemical entities,[10,11] their potential protective effects against oxidative stress,[12] and their ability to interact with biotargets related to neurodegenerative diseases.[1] This article will briefly describe some of the recent strategies that will be depicted by current results. First, the bioaccumulation of chemicals in the environment is evaluated through a new index based on *in vitro* experiments. Then, a novel classification method to clarify the antioxidant profile of compounds is determined through the combination of four standard *in vitro* assays. Finally, the identified compounds, beneficial against oxidative stress, are tested *in silico* to evaluate their potential interactions with promising targets to fight against neurodegeneration (HDACs).

Bioconcentration: A New Index Based on UHPLC Measurements

To our knowledge, the most widely used method for the *in vitro* estimation of the bioconcentration factor (BCF) of chemicals is based on the correlation between the existent BCF data and the par-

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tition coefficients obtained in n-octanol/water systems (log P_{oct}).[13] The success of this correlation is due to the complex medium characterized by the water of the aquatic ecosystem and the lipid phase of fishes used for BCF measurements. In these conditions, the hydrophobicity is the main driving force, in close analogy with the partitioning of hydrophobic chemicals in the much simpler biphasic model system. It has already been demonstrated that, according to the OECD guidelines, a classification of compounds based on their log BCF values can be performed.[14] Only compounds with log BCF values less than 3.3 would have to be considered for further development in order to avoid toxic risks. To avoid the measurements of log BCF in animals, a predictive model was also built from the bilinear correlation between log BCF values and log P_{oct} values reported in Fig. 1. In this case, only compounds with log P_{oct} values less than 5.0 would have to be considered for further development. Moreover, this model presents two main problems. First, the bioconcentration decreases when the lipophilicity is sufficiently high, a phenomenon leading to a bilinear correlation between log BCF and log P_{oct} values (Fig. 1). Secondly, the model is based on calculated lipophilicity indices: the lack of accuracy in calculated log P_{oct} values, especially for highly lipophilic compounds, generates inadequate log BCF predictions.

Previous studies demonstrated that the well-known correlation between partition coefficients (log P_{oct}) and retention factors (log k_w) on chromatographic non-polar phases can be extended to highly lipophilic compounds by using UHPLC systems.[15] These results suggested that the difficulties associated with the measurements of log P_{oct} for very hydrophobic compounds can be solved by using chromatographic systems. The retention factors can thus offer a valuable alternative to predict BCF.

In liquid chromatography, the retention factor log k, dependent on the lipophilicity of the analytes, is given by Eqn. (1):

$$\log k = \log\left(\frac{t_r - t_{delay} - \left(V_{ext}/F\right)}{t_0 - t_{delay} - \left(V_{ext}/F\right)} - 1\right) \qquad (1)$$

where t_r and t_0 are the retention time of the solute and the unretained compound, respectively, t_{delay} is the injection delay, V_{ext} the extra-column volume and F the flow rate of the mobile phase (pure water for log k_w values).

In this work, the *in vivo* bioconcentration factors of 85 compounds (log BCF values from 0.3 to 6.0 chosen from the literature) were directly correlated with log k_w values obtained with a specific UHPLC chromatographic approach developed in our laboratory that enables the inclusion of highly lipophilic compounds.[15]

To analyze all the compounds in their neutral form, three buffers with different pH values (trifluoroacetic acid/sodium hydroxide pH 2.5, acetic acid/sodium hydroxide pH 5.0 and phosphoric acid/sodium hydroxide pH 7.5) were prepared. An ionic strength of 20 mM was chosen according to Phoebus software v1.0 (Analis, Namur, Belgium). Buffer solutions were filtered through a 0.22 µm HA Millipore filter (Millipore, Bedford, MA, USA). An Acquity UPLC system (Waters, Milford, USA) including a binary solvent manager, a sample manager with an injection loop volume of 2 µL, a photo diode array (PDA) programmable detector and a column manager with oven, was used for UHPLC measurements. The system was controlled by Empower Software v2.0 (Waters, Milford, MA) and the detection performed at appropriate wavelengths (compounds λ_{max}). Retention measurements were performed on a Hypersil™ GOLD Javelin HTS stationary phase (10 × 2.1 mm ID, 1.9 µm) (Thermo Scientific Runcorn, UK) at a flow rate of 1 mL/min, and at 30 ± 0.1°C. This column was chosen since it allows a fast determination of lipophilicity.[15] The concentration of stock solutions were 1000 ppm in MeOH and the injected solutions varied from 500 to 50 ppm, depending on the UV absorbance. An injection of uracil at 40% ACN allowed t_0 to be evaluated. t_r was determined in triplicate (data not shown). Extrapolated log k_w values were measured, at least at three different percentages of methanol. These values were plotted as a function of the mobile phase composition (φ), in which the intercept at 100% water represented the log k_w value as given by Eqn. (2):

$$\text{Log } k = \log k_w - S \cdot \varphi \qquad (2)$$

where S is a constant for a given solute and fixed experimental conditions and φ the organic modifier composition.

The relation between measured log k_w and the bioconcentration descriptor is reported in Fig. 2 together with the bioaccumulation limits defined by OECD. The decrease in bioaccumulation reported for highly lipophilic compounds confers a bilinear profile to this relationship. Even if the upper limit has to be confirmed with additional experimental data, this model suggested that a chemical is not bioaccumulative if its log k_w is lower than 4.5 or higher than 8.0. Within this interval, the compound should not be retained for drug development as it could induce adverse effects on humans and environmental problems. Hence, a bioaccumulation classification of chemicals can be done with a good confidence by using experimental log k_w values measured on Hypersil™ GOLD Javelin HTS stationary phase. In that case, according to the REACH directives, no animals are used for this toxicity assessment.

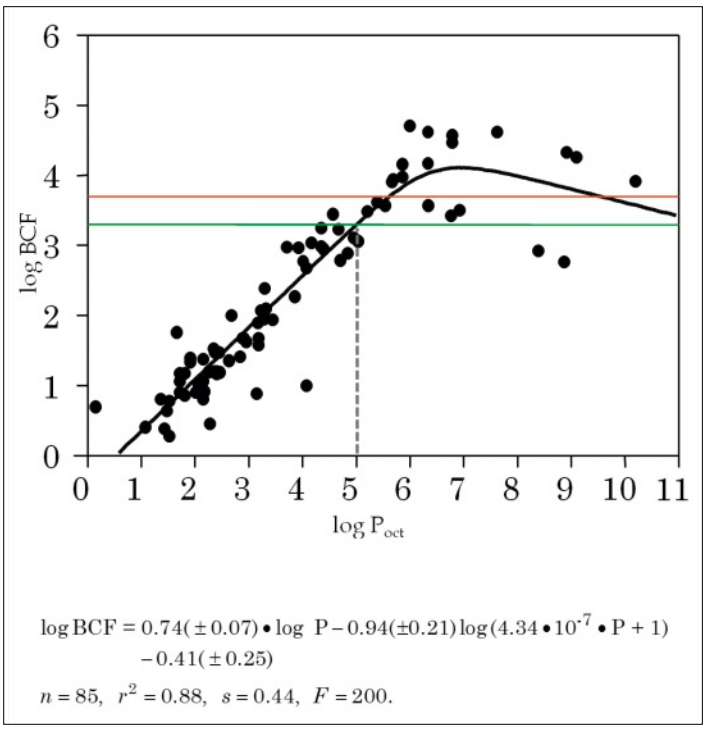

Fig. 1. Correlation between log BCF and calculated log P_{oct} values (obtained by using EPI Suite v3.12). In the equation, n is the number of compounds, r^2 the squared correlation coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses. Red and green lines represent the OECD regulatory limits: log BCF = 3.7 (very bioaccumulative) and log BCF = 3.3 (bioaccumulative), respectively. The dotted line represents the log P_{oct} value cutoff for a compound to be safely developed.

Study of the Antioxidant Profile of Chemicals

Antioxidants are of great interest against oxidative stress, because of their capability to prevent damages that often lead to pathological events.[16] In this work, four widely used antioxidant property-as-

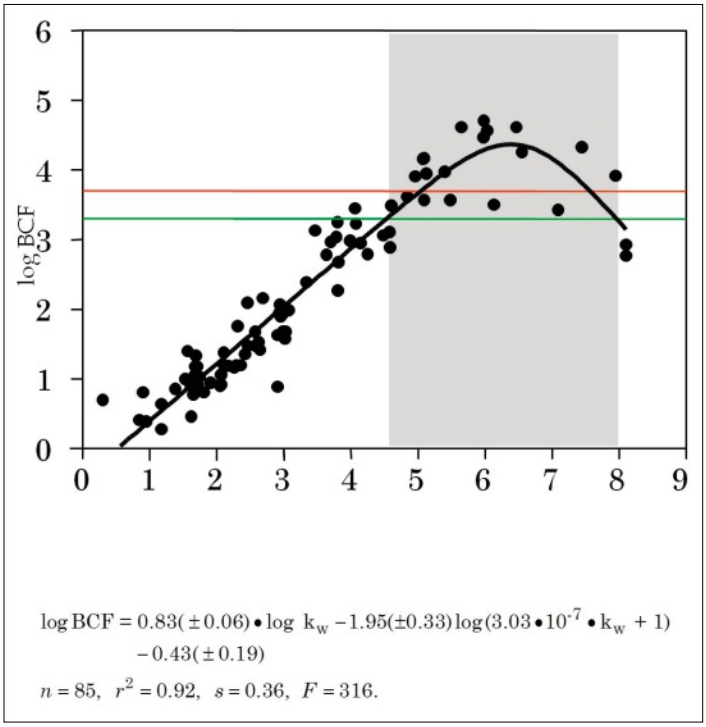

Fig. 2. Correlation between log BCF and log k_w values obtained by using Hypersil™ GOLD Javelin HTS column. In the equation, n is the number of compounds, r^2 the squared correlation coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses. Red and green lines represent the OECD regulatory limits: log BCF = 3.7 (very bioaccumulative) and log BCF = 3.3 (bioaccumulative), respectively. The gray zone represents the log K_w range in which compounds cannot be safely developed.

sessing methods were used to retrieve parameters describing the behavior of selected compounds toward reactive species of different stability. These results were combined together to finally obtain a reliable profile-based antioxidant classification.

This study was carried out on known antioxidants (ascorbic acid, caffeic acid, chlorogenic acid, gallic acid, glutathione, mangiferin, mannitol, melatonin, quercetin, resveratrol, trolox® and uric acid) purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Buchs, Switzerland) and Siegfried Handel (Zofingen, Switzerland), as the other compounds used in the following assays.

The antioxidant activity was first determined through the ALP assay, by evaluating the ability to preserve the catalytic effectiveness of the enzyme alkaline phosphatase (ALP) upon the presence of peroxyl radicals generated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The reaction followed to assess the catalytic activity of ALP was the enzymatic dephosphorylation of 4-methylumbelliferyl phosphate (MUP) to fluorescent 4-methylumbelliferone (4-MU).[12] Enzymatic hydrolysis rates of MUP were determined by a continuous spectrofluorimetric assay monitored using a Bio Tek FLX 800 microplate fluorescence reader and a Bio-Tek Power wave X microplate absorbance reader supported by KC4 v3.3 (Bio Tec Instruments Inc., Winooski, USA).

The capacity of the reference compounds to prevent the oxidative effects of radicals generated by AAPH on fluorescein was also determined by the ORAC assay.[17] The amount of fluorescence in the presence of an antioxidant compound was detected by using the same conditions and concentrations as for the ALP assay. In both tests, the ability of the compounds to protect the effectiveness of the protein was evaluated through the potency parameter pEC_{50}. This parameter, determined by a dose-response curve, represents the concentration of antioxidant compound necessary to prevent 50% of target oxidation.

In the DPPH$^{\bullet}$ and ABTS$^{\bullet-}$ assays, the capability of each compound to scavenge an initial amount of radicals was evaluated through parameters describing the antioxidant potency (ER_{50}).[18,19] The parameter ER_{50} represents the ratio of the antioxidant concentration over the radical concentration necessary to reduce 50% of radical activity. Finally, the compounds were ranked into four classes defined from the parameters obtained in the four assays: the potency toward the radicals (pEC_{50}, ER_{50}). *Class 3* comprises the most potent compounds, described by a pEC_{50} above 5.5; *class 2* includes the intermediate compounds ($5 < pEC_{50} \leq 5.5$); *class 1* is characterized by poor antioxidant compounds ($4.5 < pEC_{50} \leq 5$) whereas *class 0* has inactive compounds with pEC_{50} less or equal to 4.5 or with a percentage of protective activity of ALP and fluorescein below 15%. According to the potency (ER_{50}) values obtained with DPPH$^{\bullet}$ and ABTS$^{\bullet-}$ assays, a compound with an ER_{50} value below 0.2 has been tagged as potent antioxidant (*class 3*) while it could be considered an intermediate antioxidant (*class 2*) if its ER_{50} value is higher or equal to 0.2 and below 0.5. Finally a poor compound (*class 1*) was described by an ER_{50} value higher or equal to 0.5 and it was considered as inactive (*class 0*) if its scavenge capacity was below 15% and 10% in DPPH$^{\bullet}$ and ABTS$^{\bullet-}$ assays, respectively. Classifications of the antioxidants considered in this work are reported in Table 1: the four assays were demonstrated to be complementary for characterizing antioxidant properties and they will be used in the future for isolating and ranking new antioxidant entities according to the classification proposed herein.

Use of Antioxidants in Neurodegeneration: *in silico* Tests on HDAC2

Neuronal cells undergo functional and sensory loss when affected by neurodegenerative diseases. Oxidative stress induced by reactive oxygen species greatly contributes to this phenomenon.[20] Since antioxidants are highly desired as complementary strategies to fight against neurodegeneration, a multifunctional scenario would like such compounds to be also active on biotargets involved in neurodegenerative pathways. The histone deacetylase HDAC2 has emerged as a promising drug target for the treatment of neurodegenerative diseases.[21] The gene encoding for such a protein was found to be directly associated with modulation of memory and learning behavior. In particular, neuron-specific overexpression of HDAC2 was shown to be implicated in memory impairment, decreased synaptic plasticity and inactivation of the CREB-CBP pathway. HDAC2 also regulates CoREST, a protein known to play a role in repressing neuronal gene expression. The inhibition of this target was demonstrated to be beneficial in animal models of neurodegeneration.[3,22] Hence, to gain further insight into the possibility of antioxidants to bind HDAC2, preliminary *in silico* interaction studies were carried out.

The three-dimensional structure of HDAC2 in complex with a specific benzamide inhibitor was retrieved from the Protein Data Bank (PDB ID 3MAX). The HDAC2 active site consists of a catalytic core in which the zinc-dependent deacetylase reaction takes place, a lipophilic tube and a 14 Å long internal cavity called foot pocket immediately adjacent to the core.[23] By using the software Hermes (Cambridge Crystallographic Data Center, Cambridge UK), the ligand was extracted, the monomer A of the protein isolated and hydrogen atoms added to the final monomeric structure. Water molecules were deleted whereas the zinc atom was kept into the binding site.

A database containing the co-crystallized inhibitor and the three-dimensional

Table 1. Antioxidant activity assessed in the ALP, ORAC, DPPH®- and ABTS®-assays. Compounds are classified in classes, color-coded according to their antioxidant profile from light gray *(class 0)* to dark gray *(class 3)*.

ALP assay			ORAC assay			DPPH$^{\bullet-}$ assay		ABTS$^{\bullet-}$ assay	
Compounds	pEC_{50}	% protective activity at 10 µM	Compounds	pEC_{50}	% protective activity at 10 µM	Compounds	ER_{50} at 90 min	Compounds	ER_{50} at 90 min
Quercetin	5.95 ± 0.04	91 ± 8	Mangiferin	6.04 ± 0.06	89 ± 10	Gallic acid	0.04 ± 0.01	Gallic acid	0.06 ± 0.01
Mangiferin	5.74 ± 0.17	100 ± 9	Quercetin	5.78 ± 0.02	95 ± 3	Quercetin	0.09 ± 0.01	Quercetin	0.07 ± 0.01
Resveratrol	5.72 ± 0.03	82 ± 4	Caffeic acid	5.65 ± 0.08	89 ± 11	Chlorogenic acid	0.11 ± 0.03	Mangiferin	0.11 ± 0.01
Chlorogenic acid	5.68 ± 0.03	100 ± 7	Chlorogenic acid	5.65 ± 0.08	89 ± 11	Mangiferin	0.12 ± 0.06	Resveratrol	0.12 ± 0.02
Caffeic acid	5.66 ± 0.10	100 ± 6	Resveratrol	5.57 ± 0.04	90 ± 3	Caffeic acid	0.17 ± 0.01	Glutathione	0.17 ± 0.01
Gallic acid	5.31 ± 0.03	100 ± 2	Gallic acid	5.29 ± 0.06	85 ± 14	Trolox®	0.21 ± 0.02	Caffeic acid	0.17 ± 0.03
Melatonin	5.20 ± 0.12	50 ± 7	Melatonin	5.28 ± 0.05	51 ± 11	Ascorbic acid	0.28 ± 0.08	Uric acid	0.25 ± 0.02
Uric acid	4.89 ± 0.05	29 ± 9	Trolox®	4.74 ± 0.12	15 ± 4	Resveratrol	0.46 ± 0.08	Chlorogenic acid	0.26 ± 0.01
Trolox®	4.85 ± 0.14	34 ± 11	Glutathione	na[a]	3 ± 1	Uric acid	na[a]	Trolox®	0.28 ± 0.04
Ascorbic acid	4.51 ± 0.17	16 ± 6	Uric acid	na[a]	1 ± 1	Glutathione	na[a]	Ascorbic acid	0.30 ± 0.03
Glutathione	na[a]	14 ± 6	Ascorbic acid	na[a]	1 ± 1	Mannitol	na[a]	Melatonin	0.32 ± 0.07
Mannitol	na[a]	4 ± 4	Mannitol	na[a]	1 ± 1	Melatonin	na[a]	Mannitol	na[a]

[a]na: not active

antioxidant compounds listed in Table 1 was built by using the program SYBYL X v.1.3 (Tripos Inc., St. Louis, MO). GOLD v.5 was used to perform molecular docking studies.[24] Preset options of the GOLD genetic algorithm were chosen, allowing the exploration of all the possible ligand conformations in the HDAC2 pocket, defined by 6 Å around the co-crystallized ligand. A total of 100 docking solutions were generated for each compound, evaluated through the GoldScore scoring function.

Before studying the potential interaction between antioxidants and HDAC2, the co-crystallized ligand was re-docked onto the active site of the enzyme in order to validate the docking methodology. A single cluster containing docking solutions with RMSD values <1 Å with respect to the original pose was obtained. Moreover, the solution with the highest GoldScore corresponded to the docking pose with the lowest RMSD (data not shown).

Among the antioxidants for which possible interactions with HDAC2 were investigated, chlorogenic acid showed the most interesting results. The cyclohexylaliphatic chain lying in the lipophilic tube was found at the bottom of such a channel, partially occupying the foot pocket (Fig. 3). The zinc ion, chelated by Asp 181, Asp 269 and His 183 residues, was further stabilized by interactions with the carboxyl and hydroxyl pendant groups of the cyclohexane. Moreover, the acidic function of the antioxidant formed a salt bridge with the Arg 39 side chain. One more hydroxyl pendant group of the cyclohexane ring and the oxygen atom of the carboxyl function made polar contacts with Gln 265 and Tyr 308 side chain residues, respectively. The aliphatic chain was stabilized by van der Waals contacts with the lipophilic tube (Gly 154, Phe 155), ending with a stacking interaction between the aromatic portion of the ligand and the Phe 210 side chain, further stabilized by van der Waals contacts with Leu 276 (Fig. 3).

Molecular docking and structural analysis suggested the possibility for the chlorogenic acid to bind to the HDAC2 active site, forming a complex stabilized by an intensive hydrophobic and hydrogen bonding network. Structural analogies with general HDACs inhibitors and implication of the same amino acid residues in the binding can also confirm this hypothesis. Indeed, this result should be confirmed by *in vitro* inhibition tests. If so, the chlorogenic acid could be used as a scaffold for the conception of a new class of HDAC inhibitors, conserving also antioxidant properties against oxidative neuronal damages.

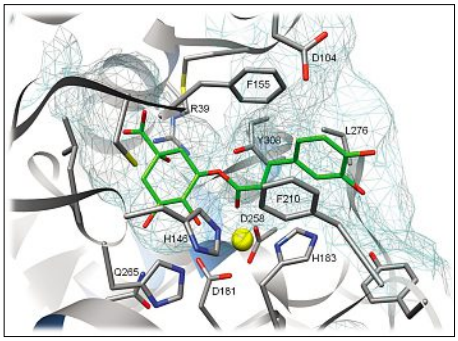

Fig. 3. HDAC2 active site in complex with chlorogenic acid (GOLD bestranked pose). The carbon atoms of the ligand, represented in stick, are colored in green whereas the oxygens are red. The zinc ion is represented as a yellow sphere. The residues of the protein (flat gray ribbons) are represented in stick and colored according to the atom type: oxygens in red, nitrogens in blue, hydrogens in gray. The light blue mesh surface defines the HDAC2 pocket where the interactions can take place.

Conclusions

Log k_w values measured through UHPLC methodology, using the Hypersil™ GOLD Javelin HTS stationary phase, was demonstrated to be promising for the estimation of bioconcentration of chemicals in the environment. As low time consuming analytical measurements were carried out, no fish species were required for bioconcentration evaluation, in line with recent legislative requirements. It was shown that compounds with log k_w values inferior to 4.5 or higher than 8.0 could be developed without any problem of bioconcentration. Such guidelines should be considered in drug design projects to avoid potential damages to human health and the environment. In line with this aim, strategies to fight against oxidative stress caused also by exposure to new chemical entities should be sought. Compounds with specific antioxidant properties are one possible strat-

egy to prevent such damage mechanisms. In this work, the antioxidant potency determined through ALP and ORAC assays (pEC_{50}) and the concentration ratio (ER_{50}) determined by the $DPPH^{\bullet}$ and $ABTS^{\bullet-}$ assays, were used to evaluate a series of antioxidant compounds. Thresholds were set up in order to rank them according to their antioxidant activity. The interest of these compounds in neurodegeneration was also highlighted *in silico* by evaluating interactions with the HDAC2 enzyme, directly involved in neurodegenerative processes. Chlorogenic acid, a highly antioxidant compound according to the proposed classification, showed important contacts with this target. *In vitro* enzymatic assays are ongoing in order to validate such observations. Moreover, the four assays determining antioxidant profiles will be performed in parallel to screen a large set of compounds for the identification of new antioxidant and potential multifunctional drug candidates.

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