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Design of Intelligent Molecules as Models for Production of New Anti-Alzheimer´s Medicines Using Epigenetic Modulation

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Alzheimer's disease is a neurodegenerative illness, so far without cure, that affects an increasing number of people around the world. Due to the adverse effects caused by drugs for treating this disease currently in clinical use, it is necessary to search for more effective and less aggressive therapeutic resources. In this work a modern technological alternative was used to build new drugs useful for the treatment of Alzheimer's disease from fungi. This strategy consists on the development of pools of fungal molecules able of act in intelligent synergistic mechanisms to block acetylcholinesterase, enzyme involved in the development of Alzheimer's disease. Expression of pools containing new secondary metabolites can be achieved by adding DNA methyltransferase inhibitors to the fermentative process. This fast *in vitro* approach was used to build a combination of fungal metabolites containing acetylcholinesterase inhibitors. Biosynthesis elicitation was achieved by adding DNA methyltransferases inhibitors (hydralazine and procainamide) to solid culture media and then filamentous fungi *Aspergillus chevalieri, Talaromyces calidicanius, Clonostachys rogersoniana, Fusarium nygamai*, and *Penicillium sp*. were added. Straightforward HPLC analysis showed elicitation of new compounds by all species, being hydralazine the most effective epigenetic modifier especially towards *T. calidicanius*. Procainamide was able to enable expression of several major new secondary metabolites as for *A. chevalieri* (RT 51 min), and *F. nygamai* (RT 21.5 min). In general, the pools of molecules expressed after epigenetic modulation were more effective in acetylcholinesterase inhibition than the metabolites expressed without modulation. Modulation of *C. rogersoniana* by procainamide increased approximately four times the biosynthesis of antiacetylcholinesterase metabolites in relation to the control. *T. calidicanius* and *Penicillium* species modulated with procainamide produced a pool of metabolites about twice more active in regarding to the basal metabolome, while the pool of metabolites from *C. rogersoniana* and *T. calidicanius* were five and three times more active (40.34 ± 0.15% and 48.54 ± 1.34%, respectively) than the non-modulated extracts.

1 Introduction

Alzheimer's disease is a neurodegenerative illness of huge social impact that affects an increasing number of people worldwide. Currently, most treatments for Alzheimer's disease are based in acetylcholinesterase inhibitors since the cure for this pathology is still unknown. Drugs currently in clinical use cause several adverse effects, being necessary to search for faster, effective and less aggressive therapeutic strategies able to minimize or delay the major symptoms of Alzheimer's disease (Dey et al., 2017). Research involving fungi represents an important role in the discovery for new secondary metabolites, including acetylcholinesterase inhibitors (Lima et al., 2018a). Fungi are microorganisms that produce a wide variety of compounds known for their biological properties. However, these organisms have silent biosynthetic gene clusters not still expressed without biotic or abiotic stimulation (Rutledge and Challis, 2015) and several approaches have been

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developed to stimulate gene expression in fungi, aiming at production of new biologically active substances (Takahashi et al., 2013).
A modern technological alternative to build new drug leads in this field is the development of pools of fungal molecules able to act in intelligent synergic mechanisms to block acetylcholinesterase, increasing acetylcholine level in the patients' brains. Expression of these pools containing new secondary metabolites can be achieved by adding histone deacetylases (HDAC) or DNA methyltransferases inhibitors (DNAMT) to the fermentative process. These chemical compounds, called epigenetic modifiers, activate the expression of silent biosynthetic pathways by fungi (Lima et al., 2018b). In addition to the production of new metabolites, this approach may also lead to an increase in the yield of compounds commonly produced under classical conditions (Williams et al., 2008), which is extremely important approach to produce metabolites of interest.
Epigenetic modulation consists in the interaction of these modulators with DNA, causing changes in gene expression, through activation of naturally silenced biosynthetic routes (Cherblanc et al., 2013; Chung et al., 2013). In addition to histone modification, DNA methylation is one of the most well-known gene regulation processes (Cherblanc et al., 2013). Methylation of DNA corresponds to an epigenetic modification governed by DNA methyltransferases, enzymes responsible for catalyzing the transferring of methyl groups to DNA (Cichewicz, 2010), leading to transcriptional repression (Cherblanc et al., 2013). Among the inhibitors of DNA methyltransferase, the most used are procainamide hydrochloride (PROCA) and hydralazine hydrochloride (HYDRA). In the present work, the modulators PROCA and HYDRA were used for metabolic modulation of some species of filamentous fungi (*Aspergillus chevalieri*, *Talaromyces calidicanius*, *Clonostachys rogersoniana*, *Fusarium nygamai*, and *Penicillium* sp.) in solid culture medium, aiming at the expression of new secondary metabolites with acetylcholinesterase inhibitory activity.

2 Materials and methods

2.1 Materials

The reagents used for analysis of AChE inhibition were TRIS/HCl by Invitrogen (Carlsbad, USA); acetylthiocholine iodide (ATCI) (Sigma, St. Louis, USA); 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) (Sigma, St. Louis, USA); acetylcholinesterase from Electrophorus electricus, type V-S (Sigma, St. Louis, USA); albumin bovine serum (Sigma, St. Louis, USA); and eserine (Sigma, St. Louis, USA). For analysis by HPLC, a Shimadzu 20A series high performance liquid chromatograph was used consisting of two LC-20AT pumps, a UV-visible SPD-20A detector, Kromazil C18 250 x 4.6 5 µm analytical column Nucleosil C18 250 x 3.0 mm, 5 µm (Supelco) and data acquisition software LC solution. Procainamide and hydralazine hydrochlorides were purchased from Sigma (St. Louis, USA). Microorganisms used in this study, *Aspergillus chevalieri* (Ac), *Talaromyces calidicanius* (Tc), *Clonostachys rogersoniana* (Cr), *Penicillium* sp. (P) and *Fusarium nygamai* (Fn), were isolated from soil at Embrapa Maize and Sorghum (Minas Gerais, Brazil), where they are deposited under the codes BRM047709, BRM047712, BRM047708, BRM047710 and BRM047711, respectively.

2.2 Epigenetic modulation

Each fungal species was grown in Petri dishes containing solid culture medium containing bacteriological agar (39 g/L) beef extract (10 g/L), glycerin (10 g/L) sterilized at 120 ºC for 20 min (control experiment). Petri dishes containing the modulators were prepared with the same culture medium. This medium was sterilized in Erlenmeyer flasks and, after cooling to 60 ºC, epigenetic modulators PROCA and HYDRA were added, separately, inside a laminar flow cabinet, to achieve a final concentration of 500 µmol/L before pouring into Petri dishes (30 mL per plate). After solidification, each fungus was inoculated in triplicate. Controls were carried out containing culture medium and the modulators (without inoculation of fungi), to measure possible spontaneous degradation of modulators in the culture medium. Fungal cultivation took place for 28 days at 25 ºC. Then, fungal metabolites were recovered using methanol. In sequence, HPLC metabolic profile and acetylcholinesterase inhibitory activity of the resulting extracts were assessed.

2.3 HPLC analysis

Fungal extracts obtained as a result of epigenetic modulation, control extracts (basal metabolism), and extracts of culture medium containing the modulators (PROCA or HYDRA) were dissolved in HPLC grade methanol (1 mg/mL). The following chromatographic conditions were used for HPLC analyses: flow 0.6 mL/min; injection volume 20 µL; temperature 25 °C; Mobile phase: HPLC grade acetonitrile (A) and ultrapure water containing 0.1% formic acid (B). Gradient method: 0.01-5 min, 95% B, 5-30 min, 50% B, 30-45 min, 0% B, 45-53 min, 0% B, using A to complete 100%. The chromatograms were monitored at 270 nm.

2.4 Acetylcholinesterase inhibition assay

This assay was carried out based on the methodology described by Ellman et al. (1961), with modifications (Dominguete and Takahashi, 2018) and performed in 96-well microplates in quintuplicate. In each well were added 25 µL of fungal extracts (10 mg/mL in DMSO), 50 µL of Tris-HCl buffer (50 mM, pH 8.0), 125 µL DTNB solution (3 mM), and 25 µL of ATCI solution (15 mM). Eserine (10 mg/mL in DMSO) was used as positive and DMSO as negative control. Absorbance was measured (405 nm, Microplate Reader) for eight times, with intervals of 1 min. Then, AChE solution (25 µL, 0.22 U/mL in buffer) was added to the wells and absorbances were measured ten times at 405 nm, with 1 min intervals. The inhibitory activity of AChE was measured following the increase of yellow color resulting from reaction of thiocholine with dithiobisnitrobenzoate ion. Percent inhibition was calculated using the formula: Inhibition (%) = 100 - [(RSsample / RBcontrol) * 100], where RSsample = rate of sample extracts reaction and RBcontrol = rate of blank.

2.5 Statistical analysis

The average percentages of inhibition of the extracts were compared by analysis of variance (single-factor ANOVA) followed by Tukey test, with 95 % confidence. Graphpad Prism 5.02 software was used for analysis.

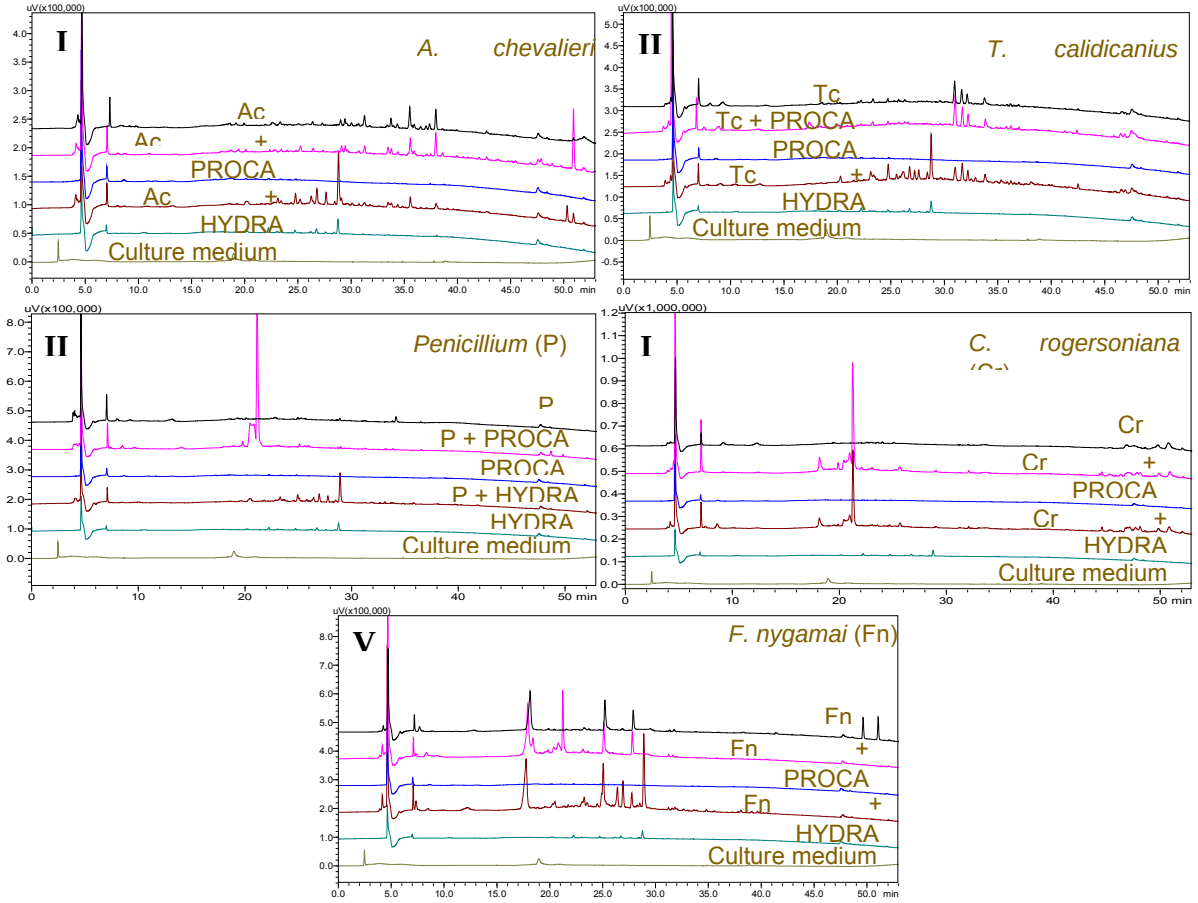

Figure 1: Chromatograms (270 nm) of profiles from the fungal extracts of A. chevalieri (I), T. calidicanius (II), Penicillium (III), C. rogersoniana (IV) and F. nygamai (V) obtained before (Ac, Tc, P, Cr and Fn) and after induced production of metabolites using procainamide (PROCA) and hydralazine (HYDRA).

3 Results and Discussion

3.1 Evaluation of epigenetic modulation via HPLC analysis

HPLC analysis showed that the addition of DNA methyltransferases inhibitors PROCA and HYDRA during cultivation of the fungal species *Aspergillus chevalieri* (Ac), *Talaromyces calidicanius* (Tc), *Clonostachys rogersoniana* (Cr), *Penicillium* sp. (P) and *Fusarium nygamai* (Fn) promoted expression of new secondary

metabolites (Figure 1). Procainamide led the species *A. chevalieri* to produce a new major metabolite (Retention time, RT, 51.0 min, Chromatogram I, Ac + PROCA), not observed in the control (Chromatogram I, Ac). Under modulation with hydralazine, this fungus produced metabolites other than those expressed in its basal metabolism (control), including a major metabolite (RT 28.5 min) and two minority ones (Chromatogram I, Ac + HYDHA) showing that the modulators activated biosynthetic routes silenced, leading to elicitation of new compounds. For *T. calidicanius*, no significant variation was observed in the metabolic profile when procainamide was used (Chromatogram II, Tc + PROCA), while modulation with hydralazine resulted in the elicitation of several different metabolites. The major new metabolite was found at RT 28.5 min, while minor new metabolites were observed in RT 24.5, 26.5, 27.0 and 27.5 min (Chromatogram II, Tc + HYDRA). The species *Penicillium* followed the same pattern, presenting a new major metabolite (RT 21.5 min, Chromatogram III, P + PROCA) when grown in the presence of procainamide while addition of hydralazine to its growth resulted in a great variation of its metabolic profile (Chromatogram III, P + HYDRA). Modulation of *C. rogersoniana* the same biosynthetic routes were activated by both modulators resulting in identical metabolic profiles, with a major metabolite with RT 20.5 min (Chromatogram IV, Cr + PROCA and Cr + HYDRA); however, the metabolites produced under modulation were different from those produced by normal metabolism (Chromatogram V, Cr). New metabolites were produced by *F. nygamai* in the presence of the epigenetic modulators procainamide (TR 21.5 min, Chromatogram V, Fn + PROCA) and hydralazine (TR 28.5, 26.6 and 27.0 min, Chromatogram V, Fn + HYDRA).

Considering the whole experiment, regardless the fungal species utilized, hydralazine provoked higher compounds elicitation in relation to the basal fungal metabolism. Although both modulators, PROCA and HYDRA act by the same mechanism, i.e., inhibiting DNA-metiltranferases, they activated different biosynthetic routes, leading to the expression of new secondary metabolites by most of the same fungal species. This efficient change in the rate of genes transcription lead to an expressive metabolic diversification, showing its efficiency for the synthesis of new bioactive metabolites by fungi.

3.2 Acetylcholinesterase inhibitory activity

Although PROCA and HYDRA provoked a significant expression of new metabolites by the fungal species, an enzymatic assay was carried out to understand the role of the new metabolites as acetylcholinesterase inhibitors (Figure 2). In fact, epigenetic modulation increased acetylcholinesterase inhibition, since extracts obtained in the presence of PROCA and HYDRA were in general more active than their respective controls (growth without the modulators), except for *A. Chevalieri*, the only one that did not exhibit increase in activity, although presented variation in its metabolic profile, with production of new compounds, as evidenced by the HPLC analysis (Figure 1, Chromatogram I).

The metabolites from *T. calidicanius* and *Penicillium* species grown under modulation with PROCA were about twice more active (30.53 ± 1.44% and 37.54 ± 0.92%, respectively) than their respective controls (14.64 ± 0.51 % and 18.32 ± 0.15%, respectively). Under modulation with HYDRA increase in activity was also observed as for *T. calidicanius* that produced a pool of metabolites about three times more active (48.54 ± 1.34 %) and for *Penicillium* twice more active (40.13 ± 0.86%) in relation to the respective controls ($P < 0.0001$). The metabolites produced by *C. rogersoniana* in the presence of PROCA showed significant increase in activity (30.88 ± 0.12 %) in relation to control (8.46 ± 0.53), while HYDRA-modulated fungus extract was even more active (40.34 ± 0.15%) than the control. Although extracts from *C. rogersoniana* obtained in the presence of PROCA and HYDRA showed very similar metabolic profiles, their effect in acetylcholinesterase inhibition varied, as HYDRA-modulated extract was significantly more active. *C. rogersoniana* is reported to produce terpenes (Dong et al., 2017) and compounds of this class were previously reported as acetylcholinesterase inhibitors (Dos Santos et al., 2017).

Therefore, it is possible that epigenetic modulation by HYDRA increased terpenoid biosynthesis, therefore justifying activity increase in comparison to the control.

Regarding the species *F. nygamai*, presence of PROCA during its development led to a significant activity increase (48.64 ± 0.48%) in relation to the control (38.16 ± 1.45%), whereas HYDRA did not present significant difference. Although is expected increase in the biosynthesis of bioactive compounds, metabolic modifications caused by epigenetic agents may be either synergistic or antagonistic (Kondo et al., 2003).

Based on these results, the expression of pools containing new secondary metabolites by addition of DNA methyltransferases inhibitors was effective for the species studied, including production of new compounds with acetylcholinesterase inhibitory activity.

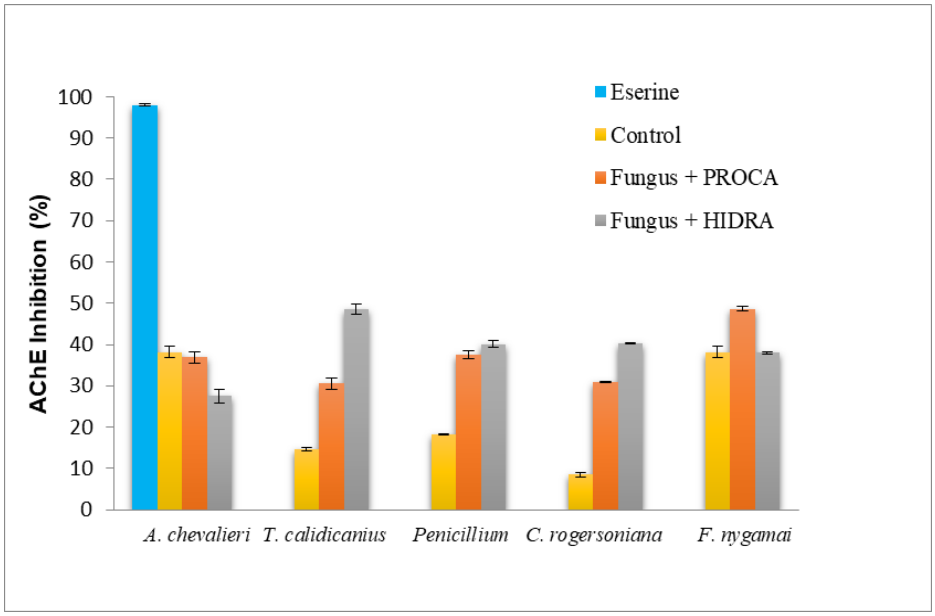

Figure 2: Acetylcholinesterase inhibitory activity of the fungal metabolites produced after modulation with procainamide (PROCA) and hidralazine (HYDRA) in comparison to respective controls.

As observed, the present approach is a prolific methodology for discovering new routes for production of new compounds efficient for increasing blood levels of acetylcholine, which is useful in the symptomatic treatment of patients with Alzheimer's disease. Classical search for bioactive fungal metabolites usually focus in the isolation and biological screening of pure compounds, which is time consuming and requires the use of large volumes of solvents. In addition, the later does not consider the synergism resulting from metabolites pools (Chear et al., 2016). On the other side, acetylcholinesterase inhibitors can also be obtained from other natural sources. Ethanol extracts from the vegetal species *Brassica oleracea* var. capitata f. rubra and *Cinnamomum verum*, for instance, inhibited acetylcholinesterase in 49.44 and 63.02%, respectively (Boğa et al., 2011) while the ethanol extract of the red algae *Grateloupia lancifolia* presented 91.1% activity (Nguyen and Kim, 2012). Anti-acetylcholinesterase activity of essential oils from plant species *Aframomum melegueta, Ocimum gratissimum* (Owokotomoa et al., 2015), and *Hyptis dilatata* (Almeida et al., 2018) are also reported. However, fungal extracts as source of new acetylcholinesterase inhibitors have the advantage of being easily engineered to activate production of bioactive metabolites in processes that can be easily scaled up (Takahashi et al., 2008).

4. Conclusions

In general, extracts from epigenetically modulated fungi, especially *C. rogersoniana* and *T. calidicanius* showed greater acetylcholinesterase inhibitory activity than un-modulated ones (control extracts). Therefore, chemically engineered fermentation using epigenetic modifiers showed good response towards the species studied. Modulation of fungal metabolism resulted in the production of pools of bioactive metabolites, being a fast-forward option for the production of novel models of acetylcholinesterase inhibitors.

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