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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

# Inhibitory activity on cholinesterases produced by aryl-phthalimide derivatives: green synthesis, in silico and in vitro evaluation

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## Abstract

**Background** Alzheimer's disease (AD) is characterized by cognitive impairment and loss of immediate memory resulting from neuronal death in different brain areas, mainly those producing acetylcholine. Acetylcholinesterase inhibitors improve cognitive function, delay mental deterioration, and reduce other symptoms. Despite being the cornerstone for treating mild–moderate AD, these compounds are only palliative agents and often have severe adverse effects. Recently, butyrylcholinesterase (BuChE) has been found to be involved in AD. The aim of this study was to synthesize a series of six phthalimides with structural relationship with monoamines and evaluate them in vitro and in silico as AChE and BuChE inhibitors. In addition, a modified version of the Bonting and Featherstone method for determining AChE activity was adapted for the assessment of BuChE activity.

**Results** Six molecules (dioxoisoindolines A–F) were synthesized in good yields using a green chemistry approach. Dioxoisoindolines E and F were more active for AChE, with a $K_i$ of 232 and 193 μM, respectively. Contrarily, dioxoisoindolines C and D showed up to fivefold greater selectivity for BuChE than AchE, with a $K_i$ of 200 and 100 μM, respectively. The competitive inhibitory activity of the latter two molecules was similar to that of the reference compounds. Molecular docking demonstrated the participation of carbonyl carbons and aromatic rings in the high affinity of dioxoisoindoles for cholinesterases.

**Conclusion** The modified version of the Bonting and Featherstone method was successfully adapted to quantify BuChE activity. Dioxoisoindolines C and D displayed greater inhibition of BuChE versus AChE, with good inhibition of both enzymes. Thus, they are promising lead compounds for developing new BuChE/AChE inhibitors.

**Supplementary information** The online version of this article (https://doi.org/10.1007/s00044-020-02543-2) contains supplementary material, which is available to authorized users.

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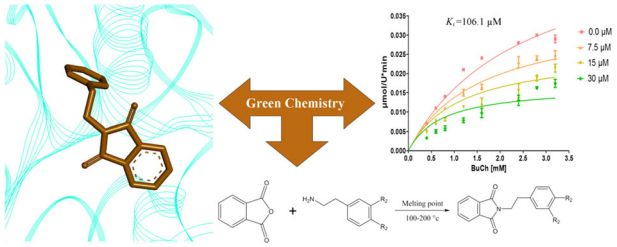
**Graphical Abstract**




**Keywords** Dioxoisoindolines · Acetylcholinesterase · Butyrylcholinesterase · Inhibitors · Alzheimer

## Highlights

- Using green chemistry principles, six phthalimides were synthesized in good yields.
- Dioxoisoindolines D and F were potent inhibitors of BuChE and AChE, respectively.
- The improved Bonting and Featherstone method proved useful for evaluating BuChE activity.

## Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disease and the most common form of dementia, is characterized by a decreased cognitive function and several behavioral abnormalities (Mohammadi-Farani et al. 2013; Liu et al. 2017). It can also be described as a brain disorder that leads to a loss of memory and cognitive abilities (Mohammadi-Farani et al. 2013). There are several hypotheses to explain the origin of AD, the most studied being the cholinergic and the β-amyloid theories (extracellular deposits in the cerebral parenchyma and intracellular neurofibrillary tangles) (Lahiri et al. 2002, 2007). An important role of the tau protein has also been proposed (Mukherjee et al. 2007).

Since the 1970s, the main strategy of many scientists doing research on AD (Ayazgok et al. 2017) has been the inhibition of the acetylcholinesterase enzyme (Si et al. 2016; Liu et al. 2017) (AChE, EC 3.1.1.7), which hydrolyzes the neurotransmitter acetylcholine in the cholinergic synapses and therefore nerve transmission ends (Kamkwalala and Newhouse 2016). More recent investigation on AD has also targeted butyrylcholinesterase (BuChE), another nonspecific cholinesterase enzyme (Wenk 2003; Liu et al. 2017; Zhang and Zhang 2017) thought to be involved in neurodegeneration, becoming a therapeutic target for the treatment of AD (Shen et al. 2017).

Currently, the treatments for AD are all based on acetylcholinesterase inhibitors (AChEIs), especially tacrine, donepezil, rivastigmine, galantamine, and memantine (Musiał et al. 2007; Guzior et al. 2015). Several studies indicate that inhibiting AChE in patients with AD contributes to improved cognitive function, delayed progression of mental deterioration, and a reduction of other characteristic symptoms (Lahiri et al. 2007). Although AChEIs are the most common treatment for mild-to-moderate AD, they do not represent a cure for the disease (Brufani et al. 1997; Huang and Mucke 2012). Moreover, those now on the market have several adverse effects. Hence, efforts are being made to look for new drugs with greater potency and less toxicity (Bajda et al. 2013).

In the search for new AChEIs during the last few decades, many techniques have been developed to measure the activity of AChE. In 1961, Ellman et al. (1961) published the ultramicro cholinesterase assay, which was an adaptation of the hydroxamic acid method for the determination of choline esters (Bonting and Featherstone 1956). Since this assay is time consuming and expensive, our group made modifications to more easily assess AChE catalytic activity (Andrade-Jorge et al. 2018b).

The catalytic site of the AChE enzyme is located inside a deep throat (Rosenberry 1975; Gilson et al. 2006; Bajda et al. 2013). It is surrounded by many other ligand recognition sites, including the oxyanionic orifice, an aromatic patch, an anionic site, and a peripheral anionic site (Dvir et al. 2010). The peripheral anionic site is intimately involved in the formation of β-amyloid peptide aggregates (Kryger et al. 1999; Colovic et al. 2013). Thus, a molecule capable of interacting at this site may have the additional benefit of stopping the aggregation of the β-amyloid peptide (Gupta and Mohan 2011).

According to recent research, isoindoline-1,3-diones are potent and novel AChEIs. They contain two phthalimide carbonyl groups that facilitate hydrogen bonding with the catalytic active site and the peripheral anionic site simultaneously. Consequently, these molecules could possibly inhibit both the formation of the β-amyloid peptide and the hydrolytic activity of AChE on acetylcholine (Mary et al. 1998; Mohammadi-Farani et al. 2013). Indeed, an isoindoline-1,3-dione was very recently crystallized and tested as an AChEI, finding good inhibitory activity ($K_i = 630$ μM) (Andrade-Jorge et al. 2018a).

Albeit if the mechanism(s) of action beyond choinesterases inhibition is unclear, the structural relationship with monoamines is attractive for exerting actions ameliorating AD progression (Farfán-García et al. 2019).

The aim of the present study was to evaluate the inhibitory activity of a series of six dioxoisoindolines on AChE and BuChE. The activity of AChE was determined with the improved Bonting and Featherstone method, as previously described. The same technique was herein adapted to measure BuChE activity. The results of the different compounds were compared in order to elucidate the affinity of dioxoisoindolines for AChE and BuChE.

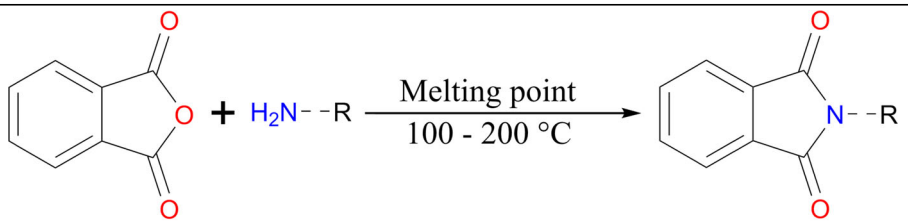
## Materials and methods

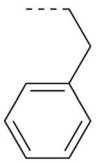
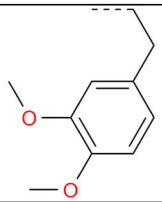
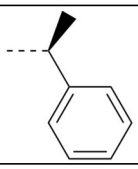
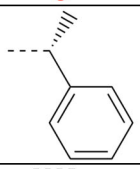
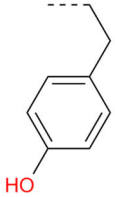
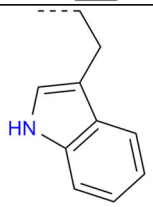
### Synthesis and characterization

All reagents and solvents were used as received from Sigma-Aldrich. Dioxoisoindolines were synthesized (Table 1) by following a published green chemistry technique, without modifications (Andrade-Jorge et al. 2017). Briefly, 1.1 mmol of phthalic anhydride and 1 mmol of the corresponding amine were placed in a 50 mL round-bottom flask. The mixture was stirred and heated to melting point (100–150 °C) for 5–10 min. Then 40 mL of ethyl acetate were added and the reaction was sonicated until a white powder formed. The precipitate was filtered, dissolved in acetone, and precipitated again with water (pH 13). The reactions were monitored by TLC (0.25 mm thick silica gel, 60 $F_{254}$ plates, Merck, Darmstadt, Germany) and the spots detected with UV light. The melting points were determined on a Stuart® SMP40 automatic melting point apparatus and are uncorrected. The products were characterized by nuclear magnetic resonance (NMR) spectra, recorded on a Varian Mercury 300 spectrometer at 300 MHz for $^{1}H$ and 75 MHz for $^{13}C$ NMR, with tetramethylsilane as internal reference. In addition, spectra were obtained on a Bruker Ascend g750 Ultrashield 750 MHz for $^{1}H$ and 189 MHz for $^{13}C$ NMR. Infrared (IR) spectroscopy was recorded on a Perkin-Elmer

**Table 1** Structures and synthetic route for isoindoline-1,3-diones

General Reaction




| ID | Substituent | Yield % | Reaction time (min) and temperature (c°) | ID | Substituent | Yield % | Reaction time (min) and temperature (c°) |
|---|---|---|---|---|---|---|---|
| DioxoisoindolineA |  | 85 | 10/150 | DioxoisoindolineB |  | 90 | 12/200 |
| DioxoisoindolineC |  | 89 | 10/100 | DioxoisoindolineD |  | 85 | 10/100 |
| DioxoisoindolineE |  | 89 | 15/250 | DioxoisoindolineF |  | 80 | 12/200 |

model FT-IR, setting spectral resolution at 4 $cm^{-1}$. Electrospray ionization high-resolution mass spectrometry was performed on a Bruker micrOTOf-Q-II instrument.

## Solutions

The phosphate buffer solution (0.1 M, pH 8) was prepared by adding 14.96 g of $K_2HPO_4$ and 1.832 g of $KH_2PO_4$ to 1000 mL of distilled water, then mixing. The sodium hydroxide solution (4.2 M) was obtained by dissolving 84.8 g of NaOH in distilled water to reach a final volume of 500 mL. For the hydroxylamine solution (2.4 M), 84.2 g $NH_2OH$•HCl was dissolved in distilled water to reach a final volume of 500 mL. For the alkaline hydroxylamine solution, equal volumes of sodium hydroxide and hydroxylamine solution were mixed immediately before use. The ferric chloride solution (0.75 M) was afforded by slowly mixing 101.55 g of $FeCl_3$•$6H_2O$ with an HCl solution (61.95%) to a final volume of 500 mL. Dissolution of 50 mg of ACh iodide in 0.63 mL of phosphate buffer provided 256 mM of stock acetylcholine solution, and 30 mg of BuCh iodide in 1 mL of phosphate buffer furnished 100 mM of stock butyrylcholine solution. Subsequently, various dilutions were made for the kinetic experiment. The stock AChE solution (10 U/mL) was prepared with the freeze-dried *Electrophorus electricus* AChE powder (EeAChE, Sigma Chemical C3389), and the stock BuChE solution (0.4 U/mL) with the freeze-dried human BuChE powder (H-BuChE, Sigma Chemical C-9971) obtained from the original source. Different concentrations of the synthesized compounds, neostigmine and galantamine, were generated by adding DMSO (<1%) or distilled water as the dissolution medium. All solutions were stored at −20 °C.

## Acetylcholinesterase and BuChE activity assay

After applying 20 µL of neostigmine or galantamine or the dioxoisoindolines to AChE, its activity and inhibition was determined with the modified Bonting and Featherstone method (Bonting and Featherstone 1956; Andrade-Jorge et al. 2018b) and the concentration–response curve was constructed. The same methodology was adapted to measure the activity and inhibition of BuChE, following the hydroxamic acid method (Fig. 1). Dilutions of BuCh were prepared at distinct concentrations (Scheme 1a), then mixed in an analog vortex mixer (Thomas Scientific). Twenty microliters of each solution was placed in a 96-well microplate, according to the pattern described in Scheme 1b. The microplate was allowed to incubate for 55 min at 37 °C in a water bath (Felisa) before stopping the reaction with 40 µL of alkaline hydroxylamine solution. After adding 100 µL of $FeCl_3$ solution to each well and premixing for 30 s, the optical density was read at 540 nm in a microplate reader (Accuris MR9600). With the corresponding values, the standard curve for BuCh was built. With the same procedure and 20 µL of BuChE in the starting mixture, the standard curve was constructed for this enzyme. The process for evaluating the activity of BuChE was repeated, adding 20 µL of one of the test compounds in each assay before adding BuChE. All assays were performed in triplicate and the data are expressed as the average of three values.

## Docking

The ligands were optimized with GaussView software 5.0.9, considering the protonation status under physiological conditions (pH 7.4). The conformational analysis was carried out on Gaussian 16 (Frisch et al. 2016) with a semiempirical method (AM1). The rotational bonds, degree of torsional freedom, atomic partial loads, and nonpolar H bonds were assigned with AutoDock 1.5.6 tools (Huey and Morris 2008). Crystal structures of the enzymes were downloaded from the Protein Data Bank (PDB: HsBuChE 4P0I, HsAChE 4PQE, EeAChE 1C2B).

The coupling conditions were programmed with AutoDock 1.5.6 tools and Raccoon (Cosconati et al. 2010), using a hybrid Lamarckian genetic algorithm (Morris et al. 1998) and an initial population of 100 randomly placed individuals. Kollmann partial charges were assigned for all proteins and Gasteiger charges for the ligands. Subsequently,

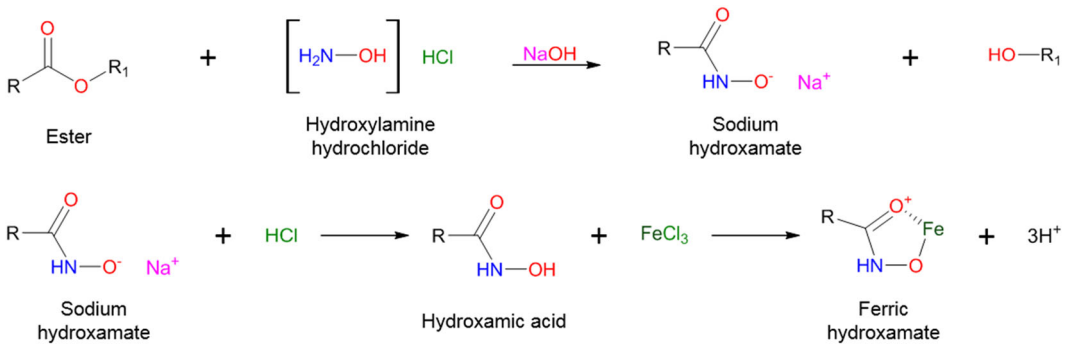
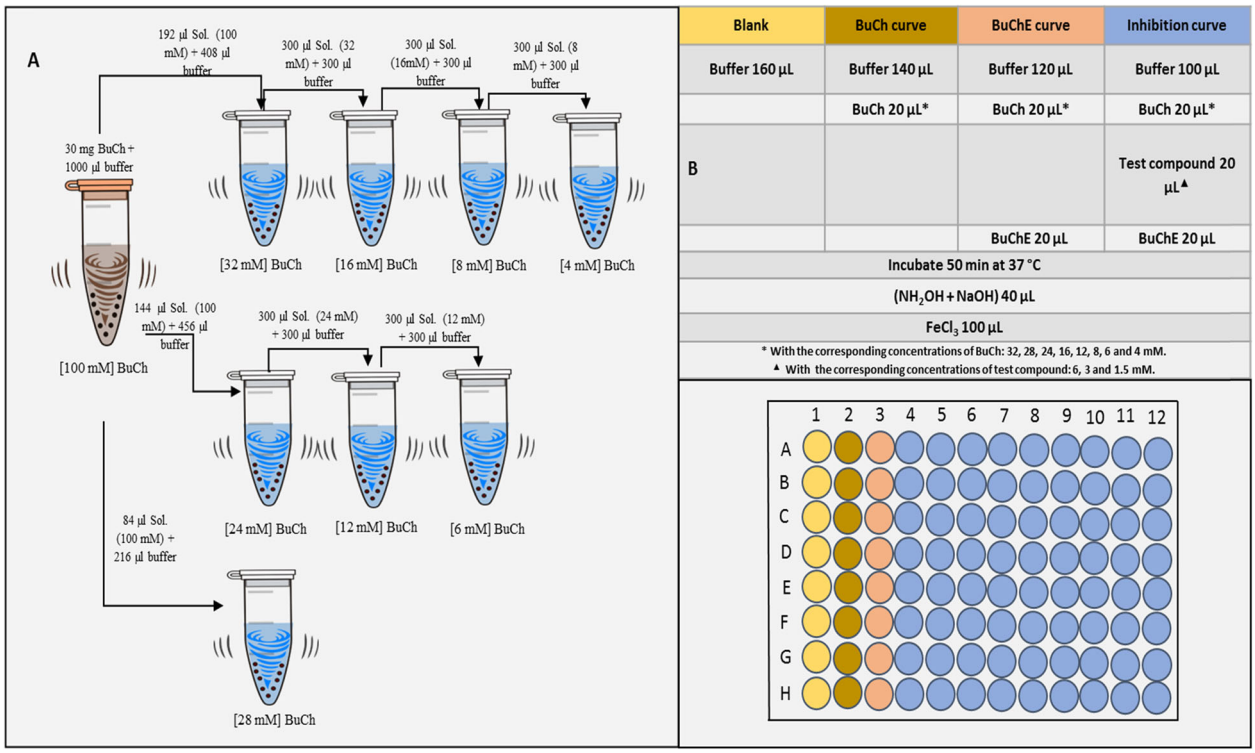



**Fig. 1** Mechanism of reaction of hydroxamic acid with the choline esters in the improved method of Bonting–Featherstone




**Scheme 1** Representation of the procedure involved in the improved method for BuChE, using a 96-well microplate for BuChE. **a** Dilutions performed for the BuCh curve. **b** The pattern used to construct the different curves to be used for evaluating the inhibitory activity of dioxoisoindolines

the grid parameter file was established for the three enzymes (HsBuChE, HsAChE, and EeAChE) with a grid of 126 × 126 × 126 Å, coordinates of $X = 138.889$, $Y = 123.537$, and $Z = 38.703$ for HsBuChE, coordinates of $X = -25.301$, $Y = 22.446$, and $Z = 0.649$ for HsAChE, coordinates of $X = 88.481$, $Y = 109.042$, and $Z = 83.026$ for EeAChE, and a mesh separation of 0.375 Å. The calculations were executed in AutoDock4 in a Linux operating system (Fedora 22).

Finally, the lowest energy state, expressed as Gibbs free energy (ΔG), was obtained for each compound. The dissociation constant ($K_d$), −log dissociation constant ($pK_d$), number of interactions and distance, and type of binding were determined with the Visual Molecular Dynamics program (VMD v.1.8.6) (Humphrey et al. 1996) and Discovery studio 4.0 client. The validation of the molecular docking was performed by reproducing the binding mode of well-known ligands (acetylcholine, neostigmine, galantamine, rivastigmine).

## Statistical analysis

The data from the cholinesterase inhibition assays are expressed as the mean ± 95% confidence intervals. Each treatment was compared with the corresponding control group. A Michaelis–Menten graph was made for nonlinear regression analysis, carried out at 95% confidence intervals on GraphPad Prism statistical software in the enzyme kinetic mode.

# Results and discussion

## Chemical characterization

Six aryl-phthalimides structurally related to some monoamine neurotransmitters were synthesized under solventless conditions, this relation is in regard to the presence of the phenylethyl or ethylamine moiety in the structures (Table 1). The complete chemical characterization of these compounds is shown below.

### 2-(2-phenylethyl) isoindolin-1,3-dione (dioxoisoindoline A)

It was obtained as a white solid, 85% yield; mp 130–131 °C (recrystallization solvent ethyl acetate); $^1$H NMR ($CDCl_3$, 300 MHz) δ (PPM) 7.82 (2H, dd, $J = 5.5$, 3.0 Hz, H-4,7), 7.70 (2H, dd, $J = 5.6$, 3.0 Hz, H-5,6), 7.32–7.18 (5H, m, H-13,14,15,16,17), 3.92 (2H, t, $J = 7.4$ Hz, H-10), 2.98 (2H, t, $J = 7.4$ Hz, H-11); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 168.1 (C-1,3), 137.9 (C-12), 133.9 (C-5,6), 132.0 (C-8,9), 128.8 (C-14,16), 128.5 (C-13,17), 126.6 (C-15), 123.2 (C-4,7), 39.2 (C-10), 34.6 (C-11). IR (ATR, $cm^{-1}$) ύ: 3031 (C–H,

Aromatic), 2935 (C–H, Aliphatic), 1705 (C=O), 1600 (C=C), 1427 ($CH_2$), 1391 (C–N). HRMS (*m/z*): 274.0752 (M + Na), calculated: 274.0838.

#### 2-(2-(3,4-dimethoxyphenyl)ethyl) isoindolin-1,3-dione (dioxoisoindoline B)

It was obtained as a light yellow solid, 90% yield; mp 171–172 °C (recrystallization solvent ethyl acetate); $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ (PPM) 7.81 (2H, dd, $J = 5.5$, 3.0 Hz, H-4,7), 7.70 (2H, dd, $J = 5.4$, 3.1 Hz, H-5,6), 6.78–6.73 (3H, m, H-13,16,17), 3.83 (3H, s, H-18), 3.80 (3H, s, H-19), 3.90 (2H, t, $J = 7.5$ Hz, H-10), 2.93 (2H, t, $J = 7.5$ Hz, H-11); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 168.2 (C-1,3), 148.7 (C-14), 147.6 (C-15), 133.9 (C-12), 132.0 (C-5,6), 130.4 (C-8,9), 123.1 (C-4,7), 120.8 (C-17), 111.8 (C-16), 111.1 (C-13), 55.7 (C-18,19), 39.3 (C-10), 34.0 (C-11). IR (ATR, $cm^{-1}$) ύ: 3063 (C–H, Aromatic), 2943 (C–H, Aliphatic), 2842 (O–$CH_3$, Aliphatic), 1705 (C=O), 1600 (C=C), 1466 ($CH_2$), 1427 ($CH_3$), 1394 (C–N), 1228 (O–$CH_3$). HRMS (*m/z*): 334.0956 (M + Na), calculated: 334.1049.

#### (S)-(2-(1-phenyl)ethyl)isoindolin-1,3-dione (dioxoisoindoline C)

It was obtained as a yellow oil, 89% yield; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ (PPM) 7.80 (2H, dd, $J = 5.6$, 2.9 Hz, H-4,7), 7.68 (2H, dd, $J = 5.6$, 2.9 Hz, H-5,6), 7.52–7.50 (2H, m, H-14,16), 7.49–7.48 (2H, m, H-13,17), 7.35–7.22 (1H, m, H-15), 5.56 (1H, q, $J = 7.3$ Hz, H-10), 1.93 (3H, d, $J = 7.3$ Hz, H-11); $^{13}$C NMR ($CDCl_3$,75 MHz) $\delta$ 168.1 (C-1,3), 140.2 (C-12), 133.8 (C-5,6), 131.9 (C-8,9), 128.4 (C-15), 127.6 (C-14,16) 127.4 (C-13,17), 123.1 (C-4,7), 49.5 (C-10), 17.5 (C-11). IR (ATR, $cm^{-1}$) ύ: 3063 (C–H, aromatic), 2935 (C–H, aliphatic), 1703 (C=O), 1600 (C=C), 1427 ($CH_2$), 1425 ($CH_3$), 1384 (C–N). HRMS (*m/z*): 274.0774 (M + Na), calculated: 274.0838.

#### (R)-(2-(1-phenyl)ethyl)isoindolin-1,3-dione (dioxoisoindoline D)

It was obtained as a white solid, 85% yield; mp 65–68 °C (recrystallization solvent ethyl acetate); $^1$H NMR (DMSO, 750 MHz) $\delta$ 7.86 (2H, dd, $J = 3.3$, 1.9 Hz, H-4,7), 7.83 (2H, dd, $J = 3.2$, 1.9 Hz, H-5,6), 7.38 (2H, d, $J = 7.6$ Hz, H-13,17), 7.33 (2H, t, $J = 7.7$ Hz, H-14,16), 7.25 (1H, t, $J = 7.3$ Hz, H-15), 5.46 (1H, q, $J = 7.3$ Hz, H-10), 1.83 (3H, d, $J = 7.3$ Hz, H-11); $^{13}$C NMR (DMSO, 189 MHz) $\delta$ 168.1 (C-1,3), 141.0 (C-12), 135.0 (C-5,6), 131.8 (C-8,9), 128.9 (C-15), 127.7 (C-14,16), 127.0 (C-13,17), 123.5 (C-4,7), 49.1 (C-10), 17.9 (C-11). IR (ATR, $cm^{-1}$) ύ: 3031 (C–H, aromatic), 2989 (C–H, aliphatic), 1704 (C=O), 1613 (C=C), 1458 ($CH_2$), 1453 ($CH_3$), 1384 (C–N). HRMS (*m/z*): 274.0761 (M + Na), calculated: 274.0838.

#### 2-(2-(4-hydroxyphenyl)ethyl) isoindolin-1,3-dione (dioxoisoindoline E)

It was obtained as a white solid, 89% yield; mp 237–239 °C (recrystallization solvent ethyl acetate); $^1$H NMR (DMSO, 750 MHz) $\delta$ 9.18 (1H, s, OH-15), 7.83–7.80 (4H, m, H-4,5,6,7), 6.96 (2H, d, $J = 8.4$ Hz, H-13,17), 6.63 (2H, d, $J = 8.4$ Hz, H-14,16), 3.73 (2H, t, $J = 7.4$ Hz, H-10), 2.79 (2H, t, $J = 7.4$ Hz, H-11); $^{13}$C NMR (DMSO, 189 MHz) $\delta$ 168.2 (C-1,3), 156.0 (C-15), 134.8 (C-5,6), 131.9 (C-8,9), 130.0 (C-12), 128.6 (C-13,17), 123.6 (C-4,7), 115.6 (C-14,16), 39.5 (C-10), 33.2 (C-11). IR (ATR, $cm^{-1}$) ύ:3255 (O–H) 3026 (C–H, aromatic), 2955 (C–H, aliphatic), 1703 (C=O), 1613 (C=C), 1466 ($CH_2$), 1384 (C–N). HRMS (*m/z*): 290.0715 (M + Na), calculated: 290.0787.

#### 2-(2-(1H-indol-3-yl)ethyl) isoindolin-1,3-dione (dioxoisoindoline F)

It was obtained as a light yellow solid, 80% yield; mp 182–184 °C (recrystallization solvent ethyl acetate); $^1$H NMR (DMSO, 750 MHz) $\delta$ 10.83 (1H, s, NH-14), 7.85 (2H, dd, $J = 5.5$, 3.0 Hz, H-7,4), 7.81 (2H, dd, $J = 5.2$, 3.1 Hz, H-5,6), 7.55 (1H, d, $J = 7.9$ Hz, H-15), 7.32 (1H, d, $J = 8.1$ Hz, H-18), 7.17 (1H, s, H-13), 7.06 (1H, t, $J = 7.5$ Hz, H-16), 6.97 (1H, t, $J = 7.4$ Hz, H-17), 3.85 (2H, t, $J = 7.5$ Hz, H-10), 3.03 (2H, t, $J = 7.7$ Hz, H-11); $^{13}$C NMR (DMSO, 189 MHz) $\delta$ 168.2 (C-1,3), 123.4 (C-4,7), 136.7 (C-20), 134.8 (C-5,6), 132.0 (C-8,9), 127.5 (C-19), 123.4 (C-13), 121.4 (C-16), 118.8 (C-17), 118.4 (C-18), 111.9 (C-12), 111.0 (C-15), 38.7 (C-10), 24.3 (C-11); IR (ATR, cm−1) ύ: 3374 (N–H) 3031 (C–H, aromatic), 2986 (C-H, aliphatic), 1703 (C=O), 1611 (C=C), 1466 ($CH_2$), 1384 (C–N). HRMS (*m/z*): 313.0869 (M + Na), calculated: 313.0947.

Three new dioxoisoindolines (dioxoisoindoline D, dioxoisoindoline E, and dioxoisoindoline F) were synthesized with the present green solventless methodology and a complete chemical characterization was made for each.

## Anticholinesterase activity assay

Previously, our group reported good inhibitory activity of dioxoisoindoline A and dioxoisoindoline B on AChE (Andrade-Jorge et al. 2018a, 2018b), but assays were not conducted at that time on BuChE. In the current contribution, six dioxoisoindolines derived from arylamines (dioxoisoindolines A–F) were evaluated for inhibition of both AChE (Fig. 25s) and BuChE (Fig. 26s). Analysis of the type of inhibition was carried out with a

Lineweaver–Burk plot. Dioxoisoindolines A, C, and D were found to be uncompetitive for AChE, while dioxoisoindolines B, E, and F proved to be competitive for the same enzyme (Table 2). For BuChE, the inhibitory activity produced by Dioxoisoindolines B–F was competitive and that by dioxoisoindoline A uncompetitive (Table 3).

Interestingly, dioxoisoindolines E and F showed the best inhibition of AChE, with a 1.5- and 2.1-fold selectivity for AChE versus BuChE, respectively. For BuChE, dioxoisoindolines C and D gave rise to the best inhibition, with a 3.5- and 5-fold selectivity (BuChE versus AChE), respectively. The latter two molecules are likely more relevant, because the ratio of these two cholinesterases changes with the onset of AD. As Savelev et al. observed, the level of AChE decreases up to 15%, while that of BuChE increases up to 90%. Therefore, a higher affinity for BuChE than AChE is a desirable characteristic for an AD medication, as evidenced by a greater improvement in the cognitive performance of patients (Savelev et al. 2004). The affinity of the rest of the molecules currently tested was very similar for both enzymes.

**Table 2** Inhibition constant, type of inhibition and Gibbs free energy obtained for the interaction of the dioxoisoindolines and two references drugs with *Electrophorus electricus* AChE

| Ligand | $K_i$ (μM) | Interval 95% | Type of inhibition | ΔG (kcal/mol) |
|---|---|---|---|---|
| Dioxoisoindoline A | 504.4 | 436.6–572.2 | Uncompetitive | −4.67 |
| Dioxoisoindoline B | 632.3 | 512–702 | Competitive | −4.53 |
| Dioxoisoindoline C | 708.3 | 623.6–729.9 | Uncompetitive | −4.46 |
| Dioxoisoindoline D | 531.2 | 558.4–700.8 | Uncompetitive | −4.62 |
| Dioxoisoindoline E | 232.6 | 202.5–262.8 | Competitive | −5.15 |
| Dioxoisoindoline F | 193.9 | 168.7–219.0 | Competitive | −5.24 |
| Galantamine | 0.316 | 0.270–0.362 | Competitive | −9.21 |
| Neostigmine | 22.56 | 20.19–24.93 | Competitive | −6.56 |

**Table 3** Inhibition constant, type of inhibition and Gibbs free energy obtained for the interaction of dioxoisoindolines and two references drugs with human BuChE

| Ligand | $K_i$ (μM) | Interval 95% | Type of inhibition | ΔG (kcal/mol) |
|---|---|---|---|---|
| Dioxoisoindoline A | 499.7 | 413.7–585.7 | Uncompetitive | −4.68 |
| Dioxoisoindoline B | 596.4 | 496.9–695.9 | Competitive | −4.57 |
| Dioxoisoindoline C | 200.5 | 174.4–226.6 | Competitive | −5.24 |
| DioxoisoindolineD | 106.1 | 76.7–135.7 | Competitive | −5.63 |
| Dioxoisoindoline E | 501.5 | 412–590.1 | Competitive | −4.68 |
| Dioxoisoindoline F | 430.2 | 347.5–512.9 | Competitive | −4.77 |
| Galantamine | 7.29 | 6.50– 8.06 | Competitive | −7.28 |
| Neostigmine | 3.74 | 3.28–4.19 | Competitive | −7.69 |

**Table 4** Gibbs free energy (ΔG), the dissociation constant ($K_d$), and $-\log_{10}$ dissociation constant ($pK_d$) for the interaction of the test and references molecules with the enzymes

| ID | AChE (*Electrophorus electricus*) | | | AChE (*Homo sapiens*) | | | BuChE (*Homo sapiens*) | | |
|---|---|---|---|---|---|---|---|---|---|
| | ΔG (kcal/mol) | $K_d$ (μM) | $pK_d$ | ΔG (kcal/mol) | $K_d$ (μM) | $pK_d$ | ΔG (kcal/mol) | $K_d$ (μM) | $pK_d$ |
| Dioxoisoindoline A | – | – | – | −7.19 | 5.35 | 5.3 | – | – | – |
| Dioxoisoindoline B | −8.93 | 0.283 | 6.5 | −5.91 | 46.77 | 4.3 | −7.27 | 4.68 | 5.3 |
| Dioxoisoindoline C | – | – | – | −5.83 | 52.95 | 4.3 | −6.12 | 32.71 | 4.5 |
| Dioxoisoindoline D | – | – | – | −5.65 | 72.18 | 4.1 | −6.41 | 20.11 | 4.7 |
| Dioxoisoindoline E | −8.27 | 0.867 | 6.1 | −6.95 | 8.11 | 5.1 | −7.37 | 3.93 | 5.4 |
| Dioxoisoindoline F | −9.06 | 0.229 | 6.6 | −5.61 | 77.44 | 4.1 | −8.22 | 0.94 | 6.0 |
| Acetylcholine | −4.52 | 462.23 | 3.3 | −4.56 | 454.93 | 3.3 | −4.87 | 268.04 | 3.6 |
| Neostigmine | −5.71 | 69.17 | 4.1 | −5.65 | 72.7 | 4.1 | −5.74 | 62.14 | 4.2 |
| Galantamine | −6.58 | 17.32 | 4.8 | −6.73 | 14.2 | 4.8 | −8.7 | 0.42 | 6.3 |
| Rivastigmine | −6.32 | 22.18 | 4.6 | −6.74 | 11.49 | 4.9 | −6.28 | 24.88 | 4.6 |

**Table 5** Amino acid residues and type of interactions involved in the approach of the ligands (phthalamides and reference molecules) to the enzymes

| | Ligand | Hydrophobic | π–π interaction | H-bond | π-cation | π-anion |
|---|---|---|---|---|---|---|
| BuChE human | Dioxoisoindoline A | – | – | – | – | – |
| | Dioxoisoindoline B | Val288,Trp82, His438 | Trp23, Phe329 | Ser198 | – | Glu197 |
| | Dioxoisoindoline C | – | Trp82 | Gly116 | – | Glu197 |
| | Dioxoisoindoline D | Pro84,Tyr114 | Trp82 | Gly116 | – | Glu197 |
| | Dioxoisoindoline E | Leu286 | Trp82,Trp231,His438 | Gly116 | – | Glu197 |
| | Dioxoisoindoline F | Gly117 | Trp82,Trp231,His438 | His438 | – | Glu197 |
| | Butyrylcholine | – | Trp231 | Ser198 | – | Glu197 (electrostatic) |
| | Neostigmine | Glu197 | Trp82,His438 | Tyr128 | – | Glu197 (electrostatic) |
| | Galantamine | Trp82 | Tyr128 | Glu197, Gly116, Thr120 | – | – |
| | Rivastigmine | Trp82 | His438 | Thr120 | – | – |
| AChE Human | Dioxoisoindoline A | – | – | – | – | – |
| | Dioxoisoindoline B | – | His447,Trp86,Trp236 | – | – | Glu202 |
| | Dioxoisoindoline C | – | – | – | – | – |
| | Dioxoisoindoline D | – | – | – | – | – |
| | Dioxoisoindoline E | Ala204 | Trp86.Tyr337,His447 | Ser203 | – | Glu202 |
| | Dioxoisoindoline F | – | Trp86,Trp236, Tyr337, His447 | – | – | Glu202 |
| | Acetylcholine | His447 | Tyr337 | Ser203 | Tyr337 | Glu202 (electrostatic) |
| | Neostigmine | Glu202 | Trp86,Tyr337 | Gly122 | – | Glu202 |
| | Galantamine | Tyr337 | Trp86 | Glu202 | – | – |
| | Rivastigmine | Ala204,Tyr337 | Trp86,Trp236 | – | – | Glu202 |
| AChE of *Ee* | Dioxoisoindoline A | – | – | – | – | – |
| | Dioxoisoindoline B | – | Trp86,Tyr337 | Ser203, Tyr337 | – | – |
| | Dioxoisoindoline C | – | – | – | – | – |
| | Dioxoisoindoline D | – | – | – | – | – |
| | Dioxoisoindoline E | – | Trp86, Tyr337 | Ser203,Phe338 | – | – |
| | Dioxoisoindoline F | – | Trp86,Tyr124 | Asp74 | – | – |
| | Acetylcholine | Tyr124 | His447 | Ser203,Trp86 | Tyr337 | Glu202 (electrostatic) |
| | Neostigmine | Trp86 | Trp86 | Ser125 | Tyr337 | Glu202 (electrostatic) |
| | Galantamine | Tyr124 | Trp286,Tyr341 | Ser203,Arg296 | – | – |
| | Rivastigmine | Ile451 | Trp86 | Ser203 | – | – |

Another important aspect is the apparent better inhibition of both enzymes generated by the R-configuration of the molecules, although no significant statistical differences were found; another aspect is that dioxoisoindolines C and D have different behavior in both enzyme, this is interesting while for *Ee*AChE the inhibition is uncompetitive for *Hu*BuChE is competitive, this could be related to the molecular structure because of the disposition of the phenylethyl moiety in molecules C and D is different than molecule A, moreover, we need to take into account that both compounds possess chiral carbons that may make interact with the enzymes in a different way. More research is needed on the inhibitory potential of the R- and S-configuration. According to the data from the in vitro assays, all six dioxoisoindolines turned out to be good inhibitors of AChE and BuChE, having good affinity and selectivity in some cases. Hence, they can be considered as dual inhibitors. Finally, the reference compounds (galantamine and neostigmine) were assayed to validate the experiments, finding their $K_i$ values and

type of inhibition to be in agreement with previous reports.

## Molecular docking and theoretical calculations

To provide insights into the in vitro results and to extrapolate the findings to physiological conditions since in vitro experiments required pH = 8, a molecular docking study was carried out with the dioxoisoindolines that showed competitive behavior. As is well known, it is not possible to use molecular docking in cases of uncompetitive inhibition. Apart from furnishing essential information on the binding mode, the molecular docking data on the energy state of the ligand allowed for an analysis of its correlation to the inhibition of the enzyme. Accordingly, the values were determined for Gibbs free energy as well as the dissociation constant of the ligand–enzyme interactions in physiological conditions (pH = 7.4), comparatively with the in vitro results Gibbs free energy in the in silico results is higher, this may indicate that under physiological conditions the compounds may have greater affinity and efficacy. *Homo sapiens* AChE was presently included to extrapolate the findings to patients (Table 4), interestingly, Gibbs free energy was higher with *Homo sapiens* than EeAChE. Finally, the mean affinity for H-BuChE is very similar to that obtained for human AChE, so it is expected to have a comparable activity with the human enzyme in physiological conditions.

The interactions of the test and reference compounds with the main residues in the catalytic site of AChE and BuChE were closely observed (Table 5). Many of the interactions are due to the carbonyl carbon in the structure of the dioxoisoindolines, as described in other reports (Mohammadi-Farani et al. 2017). Moreover, hydrophobic interactions involve the aromatic groups (Figs. 2, 3, and 29s). The interactions of most of the six dioxoisoindolines with amino acid residues and their binding modes in the catalytic site were similar to those found for the reference drugs. Docking simulations were run on reference molecules (acetylcholine, butyrylcholine, neostigmine, rivastigmine, and galantamine) to reproduce the binding mode and validate the results with the six dioxoisoindolines (Figs. 27s, 28s, and 30s).

On the other hand, the analysis of the clustering of the docked ligands shown that many orientations are focused on the catalytic site of the enzymes (AChE and BuChE), displaying an around 68–100% of all conformations in the catalytic site. Moreover, the binding mode of the different ligands is very similar between them, as is shown in Figs. 2e and 3e.

## Conclusions

A series of phthalimides were synthesized in good yields by using a green chemistry approach. A fast and inexpensive method for evaluating the inhibition of ligands on AChE activity was adapted for assessing BuChE activity. The test molecules displayed good dual inhibition of AChE and BuChE. Two compounds (dioxoisoindolines C and D) showed up to fivefold selectivity for BuChE over AChE. Considering the increased presence of the former enzyme after the onset of the disease, these two compounds could be advantageous as a medication for AD. Docking results clearly demonstrate that the dioxoisoindolines interact preferentially with the catalytic site of the enzymes, which coincides with the data from the in vitro assays. In addition, carbonyl carbons play an important role in the recognition of the ligands by the enzymes. Some dioxoisoindolines presently tested hold promise as lead compounds for designing new dual cholinesterase inhibitors for AD

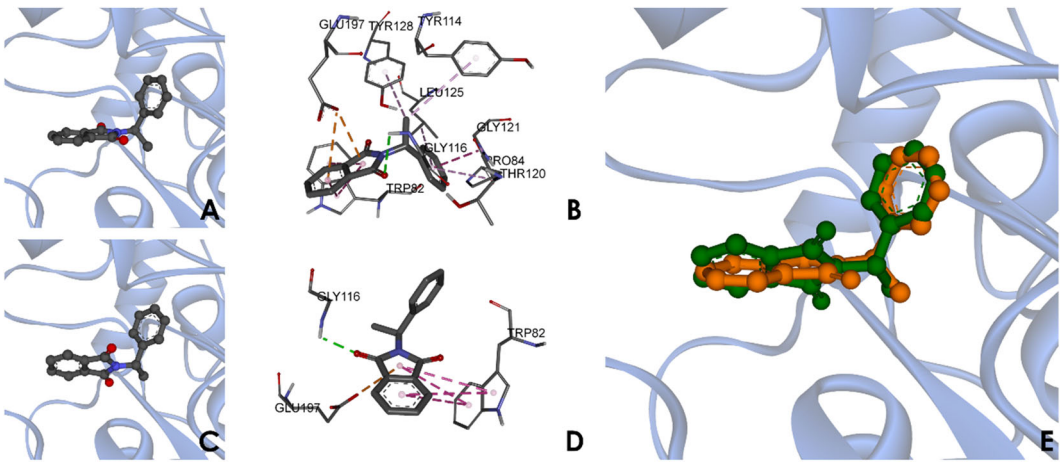



**Fig. 2** Binding mode of two representative compounds with *Homo sapiens* BuChE (PDB ID:4P0I), according to the docking simulations. **a**, **b** Lowest energy conformation of dioxoisoindoline C and its corresponding amino acid residues. **c**, **d** Lowest energy conformation of dioxoisoindoline D and its amino acid residues. **e** Binding mode of the two dioxoisoindolines, observing a great similarity in the arrangement of the molecules

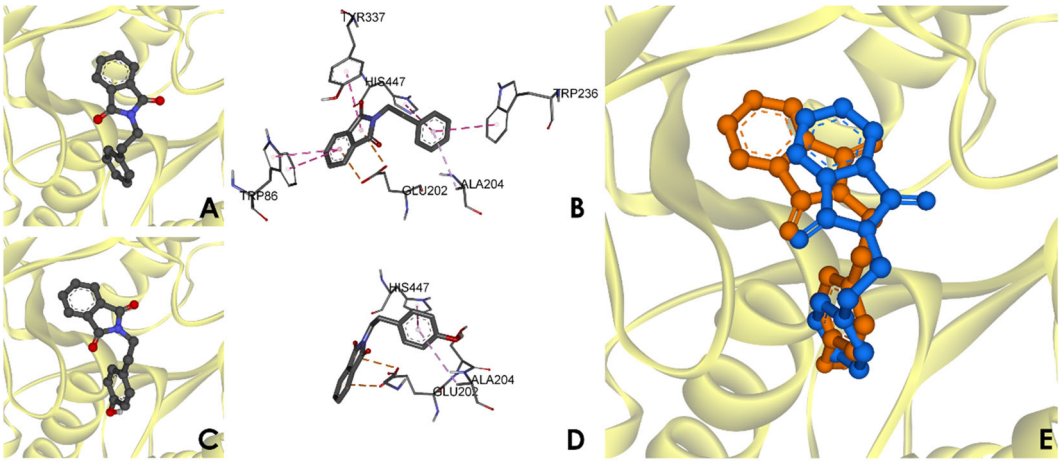



**Fig. 3** Binding mode of two representative compounds with *Homo sapiens* AChE (PDB ID:4PQE), according to the docking simulations. **a**, **b** Lowest energy conformation of dioxoisoindoline A and its corresponding amino acid residues. **c**, **d** Lowest energy conformation of dioxoisoindoline E and its amino acid residues. **e** Binding mode of the two dioxoisoindolines, observing a great similarity in the arrangement of the molecules

treatment. Furthermore, the reported phthalimides have structural relationship with monoamines, compounds which are actives as neurotransmitters or in the biotransformation of them. Additional studies are required to test these possibilities.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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