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Indomethacin-omeprazole as therapeutic hybrids? Salt and co-amorphous systems enhancing physicochemical and pharmacological properties

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ABSTRACT

Multidrug therapeutic hybrids constitute a promising proposal to overcome problems associated with traditional formulations containing physical mixtures of drugs, potentially improving pharmaceutical and pharmacological performance. Indomethacin (IND) is a non-selective non-steroidal anti-inflammatory drug (NSAIDs) that acts by inhibiting normal processes of homeostasis, causing a series of side effects, such as gastrointestinal symptoms. Proton pump inhibitors, such as omeprazole (OME), have been used to treat such gastrointestinal tract symptoms. In this work, two new multidrug therapeutic hybrids were prepared (an IND:OME salt and an IND:OME co-amorphous system) by ball mill grinding crystalline IND and OME under different conditions, i.e., liquid assisted grinding (LAG) with ethanol and dry grinding, respectively. The crystalline salt returned to a neutral state co-amorphous system when submitted to ball mill grinding in the absence of solvent (dry grinding), but the reverse process (LAG of the IND:OME co-amorphous system) showed partial decomposition of OME. The IND: OME co-amorphous system showed a higher physical stability than the neat IND and OME amorphous materials (with an amorphous stability longer than 100 days, compared to 4 and 16 h for the neat amorphous drugs, respectively, when stored at dry conditions at room temperature). Furthermore, OME presented a higher chemical stability in solution when dissolved from a salt form than from the pure crystalline form. The dissolution studies showed a dissolution enhancement for IND in both salt (1.8-fold after 8 h of dissolution) and co-amorphous (2.5-fold after 8 h of dissolution) forms. Anti-inflammatory activity using a mice paw oedema model showed an increase of the pharmacological response to IND at a lower dose (~5mg/kg) for both IND:OME salt (2.8-fold) and IND:OME co-amorphous system (3.2-fold) after 6 h, when compared to the positive control group (IND, administered at 10 mg/kg). Additionally, the anti-inflammatory activity of both salt and co-amorphous form was faster than for the crystalline IND. Finally, an indomethacin-induced gastric ulceration assay in mice resulted in a higher mucosal protection at the same dose (40 mg/kg) for both IND:OME salt and IND:OME co-amorphous system when compared with crystalline OME.

1. Introduction

The design of multidrug therapeutic hybrids combining two or more drugs into a new solid form has attracted significant research interest in the past years (Zhou et al., 2019). Drug-drug combinations can provide a better biopharmaceutical performance of the drug components, resulting in potential advantages due to possible additive or even synergistic effects. For patients, these improvements can bring benefits ranging from a higher comfort during ingestion, to a decrease of possible side effects (Arnfast et al., 2017; Singh et al., 2021). In addition, this approach also offers a low risk and low-cost path to new medicines based on drugs that are already on the market, improving their properties by the addition of a suitable therapeutically effective component without any chemical modification (Wang et al., 2021).

The success of some drug-drug combinations boosting clinical effectiveness and/or physicochemical properties of one or even both drugs, highlights the potential of continuing to explore new solid forms of multidrug therapeutic hybrids. Celecoxib-tramadol co-crystals (Cebrecos et al., 2021) were approved by the U.S Food and Drug Administration in 2021 (Viscusi et al., 2023) and provide multimodal
analgesia in a single dosage form by combining complementary analgescic and anti-inflammatory mechanisms of action. Norfloxacin-sulfathiazole salt (Gopi et al., 2016) enhanced the solubility of the drugs in different pH buffers and improved the inhibition of bacterial and fungal species relative to the parent drugs. Indomethacin-paracetamol (Faed and Demirel, 2021) co-amorphous systems effectively enhanced the solubility of indomethacin (IND) in biorelevant medium (higher than 1.6-fold) and in phosphate buffer (higher than 2.4-fold), as well as showing improvements in physical stability compared to the individual amorphous drugs.

IND (Fig. 1a) is a potent well-documented non-steroidal anti-inflammatory drug (NSAID) that despite presenting rapid absorption/permeability, is poorly water-soluble (Class II in the biopharmaceutics classification system) (Lucas, 2016; Nascimento et al., 2021). Furthermore, IND is a non-selective cyclooxygenase (COX) inhibitory agent, i.e. the inhibition occurs in both COX-1 and COX-2 isoforms. COX-2 is activated at the sides of inflammation, but COX-1 is constitutive and regulates normal body functions, such as in the gastrointestinal (GI) system. Thus, IND promotes gastric disorders, such as excessive acid production, causing mucosal injury and ulcers, which can lead to serious clinical disturbances (Badri et al., 2016; Wongrakpanich et al., 2018).

NSAIDs are often co-administered with proton-pump inhibitors, which act by reducing gastric acid production, selectively inhibiting the H⁺/K⁺ ATPase enzyme from parietal cells located in the secretory membrane of the stomach, and are widely used for therapy of several disorders related to the GI tract (Ronchi et al., 2019). Omeprazole (OME) (Fig. 1b) is one of the most effective proton-pump inhibitors in the treatment of acid-related diseases (Liu et al., 2022) leading to a long-lasting inhibition of gastric acid secretion. Despite its effectiveness, OME presents physiochemical stability issues, being decomposed when in contact with light, heat, moisture and solvents (Ronchi et al., 2019).

In this work we investigate the formation of new multicomponent solid forms of IND and OME prepared by ball milling under liquid assisted grinding (LAG) conditions with ethanol and dry grinding. Structural characterization and physicochemical properties were determined as well as the pharmacological activities of the drugs via in vivo assays, for the prepared IND:OME multicomponent solid form therapeutic hybrids.

2. Materials and methods

Materials

Indomethacin and omeprazole were purchased from Merck Denmark (Søborg, DK) and used as received. Ethanol (analytical grade) was obtained from VWR chemicals (Søborg, DK).

2.1. Preparation of multicomponent solid forms

The salt and the co-amorphous form of IND:OME were obtained by LAG with ethanol, and dry grinding, respectively. Physical mixtures of IND (γ form) (Survase et al., 2013) and OME (form A) (Ohishi et al., 1989) at a 1:1 M ratio (300 mg) were prepared by gentle mixing with a pestle and mortar for 1 min. The samples were filled into 5 mL stainless steel jars and placed in a horizontal vibrational ball mill (Mixser mill MM 400, Retsch GmbH & Co., Haan, Germany), with one 5 mm diameter stainless steel ball. The milling experiments were performed under LAG (with 60 μL ethanol) and dry grinding conditions, using a frequency of 30 Hz, at different milling times (10, 20, 30, 40, 50 and 60 min). To evaluate the possible formation of IND and OME polymorphs, both crystalline starting materials were also subjected to LAG in the presence of 60 μL of ethanol at different milling times (10, 20, 30, 40, 50 and 60 min), using a frequency of 30 Hz. The formation of the new IND:OME multicomponent solid forms was monitored by PXRD (for each milling time) and found to be completed at 40 min for LAG in ethanol and 60 min for dry grinding. LAG was additionally performed for IND:OME mixtures at other molar ratios (0.1:0.9 to 0.9:0.1). The obtained multicomponent solid forms were kept at room temperature in a desiccator in the presence of anhydrous calcium chloride as a drying agent.

Pure drug amorphous starting materials were prepared by dry grinding (for 90 min) of the individual crystalline drugs, for comparison.

2.2. Thermal analysis

Thermogravimetric analysis (TGA) was performed using a Discovery TGA (TA Instruments, New Castle, DE, USA) under ambient air atmosphere with a flow of 50 mL min⁻¹ at a heating rate of 10 °C min⁻¹. Approximately 10 mg of each sample was placed in platinum pans. Mass loss and onset temperature of mass loss were determined using Trios software (TA Instruments, New Castle, DE, USA).

Differential scanning calorimetry (DSC) was carried out using a Discovery DSC (TA instruments, New Castle, DE, USA) under a 50 mL min⁻¹ N₂ gas flow. Approximately 3 mg of the samples were transferred to a Tzero aluminum crucible with Tzero perforated lids and heated from 30 to 200 °C at a heating rate of 10 °C min⁻¹. The glass transition (T𝑔, midpoint) and melting point temperatures (m.p.) were determined using TRIOS software (TA Instruments, New Castle, DE, USA).

Images of the compounds during DSC were collected on a DSCI stare system equipment (Mettler-Toledo, Schwerzenbach, Switzerland) coupled to an OLYMPUS digital camera (model SC30, incorporating a 3.3 megapixel CMOS sensor, and an optical sub-assembly mechanic Navitar 1-6232D with 6.5X zoom) (Olympus, Walpole, MA, USA).
2.3. Powder X-ray diffraction (PXRD)

PXRD patterns were obtained using a X’Pert PANalytical PRO X-ray diffractometer (PANalytical, Almeio, The Netherlands), using Cu Ka radiation (λ = 1.54187 Å), with an acceleration voltage of 45 kV and current of 40 mA. The samples were placed on a plate and scanned from 5° to 35° 2θ in reflection mode, with a scan rate and scan step of 0.0625°/s and 0.026°, respectively. Bragg–Brentano parafocusing geometry was used. The data were collected and analyzed using the software X’Pert Data Collector (PANalytical, Almeio, The Netherlands).

2.4. Synchrotron X-ray total scattering and powder X-ray diffraction (PXRD)

X-ray total scattering was collected for amorphous forms of IND and OME and IND:OME co-amorphous systems at the beamline P02.1, PETRA III at the DESY synchrotron in Hamburg, Germany. The samples were inserted in a 0.7 mm glass capillary and sealed. A beam size of 0.2 by 0.2 mm² with a fixed high energy of 60 KeV (λ = 0.207 Å) was used. A 2D detector measuring 40 by 40 cm with a sample to detector distance of 75.4 mm was used. The collected X-ray total scattering data was integrated and converted into S(Q) and F(Q) (Si, Fig. S1) using PDFgetX3 software (Juhás et al., 2013). The background scattering signal from the empty glass capillary was subtracted from the data. A Fourier transformation of the data into the pair distribution function (PDF) was performed using PDFgetX3 (Juhás et al., 2013). A Qmin = 0.5 Å⁻¹, Qmax = 15 Å⁻¹, Qmaxint = 13 Å⁻¹ and rpol = 0.9 Å were used as parameters for the data reduction.

High-resolution powder X-ray diffraction data for IND:OME salt was collected using the same beam size and fixed high energy as for the X-ray total scattering data collection. A sample to detector distance of 375.4 mm was used. A conversion from λ = 0.207 Å to λ = 1.542 Å was performed in order to facilitate the comparison of the data with Bragg–Brentano data collected with the X’Pert PANalytical PRO X-ray diffractrometer (PANalytical, Almeio, The Netherlands). Attempts for indexing the powder pattern using TOPAS were performed and are presented in SI, Fig. S5 and S6. Unfortunately, no reasonable unit cell could be obtained to proceed for structure solution.

2.5. Infrared spectroscopy (FTIR)

Infrared spectra (IR) of crystalline IND, OME and IND:OME salt as well as of the amorphous forms of IND, OME and IND:OME were obtained using a Nicolet is10 FTIR spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) operated by using OMNIC 9.11 software, with an ATR accessory integrating a Ge window. The IR spectra were recorded with 32 scans per spectrum at a resolution of 4 cm⁻¹.

2.6. Molecular dynamics (MD) simulations of IND:OME co-amorphous system

MD simulations of IND:OME co-amorphous systems were performed with the GROMACS 5.1.1 software package (Abraham et al., 2015) using the Gaff2 force field (Abraham et al., 2015). 50 molecules of IND and 50 molecules of OME were randomly inserted in a simulation box using the ‘insert-molecules’ tool of GROMACS (Abraham et al., 2015). An energy minimization followed by NVT simulation (constant particle number [N], constant volume [V], and constant temperature [T]) during 100 ps was performed. A production stage using the NPT approach (constant particle number [N], constant pressure [P], and constant temperature [T]) for 20 ns at a time step of 2 fs was used. The simulation temperatures started at 25 °C (298.15 K) followed by a temperature increase to 80 °C (353 K) at steps of 5 °C. At each temperature increment, a NPT simulation was performed for 20 ns followed by a NPT equilibration at the final temperature of 80 °C (353 K).

The MD simulations were performed in triplicate. The Lennard-Jones potential to describe dispersion/repulsion forces and the point charge Coulomb potential for electrostatic interactions were used to calculate the intermolecular energy between pairs or neighboring atoms. Long-range electrostatic interactions were accounted for using the particle-mesh Ewald method (Darden et al., 1993), with a cutoff of 10 Å for the real space part of the interactions. A cutoff radius of 1.2 nm for the Lennard-Jones potential, and long-range dispersion corrections were added to both energy and pressure. The last 10 ns of the simulations were used to calculate the PDF, using the DiffPy-CMI program (Juhás et al., 2015), and to determine the radial distribution functions.

2.7. Physical and chemical stability studies

The physical stability of the amorphous drugs and co-amorphous system was determined by PXRD, daily or every 25 days, respectively. Amorphous forms of IND and OME, and IND:OME co-amorphous system were kept in a desiccator at room temperature in the presence of anhydrous calcium chloride as a drying agent.

Chemical stability was evaluated by high-performance liquid chromatography (HPLC) using a 1260 Infinity instrument (Agilent Technologies, Santa Clara, CA, USA). A Discovery C18 column (15cmx4.6mmx5um) was used with a mobile phase of phosphate buffer pH 7.4 and acetonitrile (60:40), respectively. The flow rate was 1 mL min⁻¹, the injection was 20 µL, and the wavelength was set at λ = 254 nm where the absorbance value of OME is detected.

2.8. Dissolution studies

Dissolution studies were carried out using a MicroDiss Profiler™ (Pion Inc., Billerica, MA, USA), with an in-situ fiber optic UV monitoring system. Absorbance was measured at 350 nm. A suspension of each sample (10000 µg of both IND:OME salt and co-amorphous system and 5173 µg of crystalline IND) was placed in a glass tube with 10 mL of phosphate buffer at pH 7.2, and stirred at 150 rpm and room temperature using a cross-type stirring magnet. The analyses were performed in triplicate over a range of 8 h under sink conditions.

The area under the curve (AUC) was calculated through the linear trapezoidal method in the considered range of time (0–8 h). The AUC values of both IND:OME salt and co-amorphous system were divided by the AUC value of crystalline IND to determine the equivalence of the samples (Bordignon et al., 2017). Drug dissolution rates (Dc) during the first 10 min of dissolution were calculated by the amount of dissolved drug (in µg) per minute, according to equation (1) (Saeedi et al., 2011):

\[ DR = (M_x/D)/1000 \]

where M is the total amount of IND in the samples (5173 µg) and D represents the percentage (%) of dissolved drug during the first 10 min.

2.9. In-vivo assays

Carrageenan-induced paw oedema in mice

The anti-inflammatory activity of IND:OME salt and IND:OME co-amorphous system was evaluated using a mice paw oedema model. The chronic inflammatory process was induced in the paw of male Swiss albino mice (weighing 20–22 g) by injecting 100 µL of 1 % carrageenan solution. Animals were split into 4 groups for oral administration of the compounds (10 mg/kg), i.e., a negative control group (vehicle, phosphate buffer pH 7.0), a positive control group (IND), and two groups receiving the IND:OME salt and IND:OME co-amorphous system. Increase in paw thickness was measured as the difference in paw thickness at “0h” (before carrageenan injection) and paw thickness after 1, 2, 3, 4, 5 and 6 h. The decrease in inflammation paw oedema was calculated (%) by taking the difference of the control-basal and the test-basal groups (Abucafy et al., 2018). Data were analyzed using GraphPad Prism v. 6.0 and an ANOVA test was performed followed by Dunnett’s test (α = 0.05).
Abuçafy et al., 2018). The study was performed in agreement with the Guidelines for Ethical Principles of the National Council for Animal Experiments Control (CONCEA) and approved by the local ethics committee for animal care and use (Protocol CEUA/FCF/CAr N° 02/2023).

2.9.1. Indomethacin-induced gastric ulcer in mice

Thirty male Swiss-Webster mice (weighing 20–22 g) obtained from the animal breeding facility of São Paulo State University (UNESP, Botucatu, SP, Brazil) were used to test the potential of IND:OME salt and IND:OME co-amorphous system to protect the stomach from gastric ulcers caused by pure IND.

Prior to the treatments, the animals were fasted overnight. Then, the animals received each treatment (described below) by oral gavage (phosphate buffered saline solution at pH 7.4) at a volume of 10 mL/kg. 6 h later, the animals were euthanized, the stomachs carefully removed and opened along the greater curvature. A saline buffer solution at pH 7.4 was used to wash the stomach before measuring the lesions through photographing the ulcer length (in mm) using the software ImageJ (National Institutes of Health, Bethesda, MD-USA).

The animals were divided into five groups (n = 6) as follows: a negative control group, which received vehicle (phosphate buffered saline at pH 7.4); a positive control group, which received pure IND (40 mg/kg); the omeprazole-treated group, which received pure OME (40 mg/kg) followed by IND (40 mg/kg); the ST1-treated group, which received IND:OME salt (80 mg/kg) and the ST2-treated group, which received the IND:OME co-amorphous system (80 mg/kg).

All experiments were conducted according to the ethical principles of the National Council for Animal Experiments Control (CONCEA), based on NIH Guidelines for the Care and Use of Laboratory Animals, and approved by the local ethics committee for animal care and use (Protocol CEUA/FCF/CAr N° 12/2023).

3. Results and discussion

3.1. Preparation and characterisation of the IND-OME salt

IND and OME in crystalline form at a 1:1 M ratio were subjected to LAG in the presence of ethanol. PXRD patterns of the milled material were obtained after different milling times (Fig. 2) and showed the formation of a new crystalline phase already after 10 min of ball mill grinding under LAG conditions, albeit with a small contamination of the crystalline starting materials. Longer milling times (up to 60 min) were used to evaluate the complete formation of the new crystalline phase. However, whilst the position of the new diffraction peaks did not change during LAG, a decrease in their intensity (possibly due to amorphization) was observed after 40 min of grinding, with a small contamination of the starting materials still present. Therefore, the formation of the new phase was considered completed after 40 min, where a higher peak intensity was observed. It should be noted however, that the new form was not pure, as some contamination of the starting materials was still visible. Moreover, the halo underneath the diffraction peaks shows that an amorphous fraction is present, potentially coming from IND as there are more diffraction peaks visible of pure crystalline OME than pure crystalline IND.

The application of mechanical energy in powders during milling can often induce polymorphic transformations, leading to the observation of new PXRD patterns (Dupont et al., 2022). To confirm that the new crystalline phase is not a result of the formation of IND or OME polymorphs, both starting materials were individually subjected to LAG in ethanol at different milling times. The results show that no phase conversions/polymorphic formation were detected during LAG of crystalline IND and OME, and these remained in their respective starting polymorphic forms, i.e., the γ form for IND (Surwase et al., 2013) and form A for OME (Ohishi et al., 1989) (Fig. 2b and 2c), thus supporting the formation of a new crystalline phase composed of both IND and OME (Fig. 2a). Furthermore, a comparison of the PXRD data of the IND:OME crystalline phase with the known IND polymorphs and the methanol solvate of IND indicates that the observed new peaks do not correspond...

Fig. 2. PXRD patterns of (a) IND:OME crystalline phase, (b) crystalline IND and (c) crystalline OME collected every 10 min for up to 60 min, using LAG in presence of ethanol.
to any known IND crystalline phase, therefore supporting the formation of a new crystalline phase (Fig. S2).

An attempt to obtain a pure IND:OME crystalline phase was conducted using LAG with ethanol by varying the IND:OME molar ratio (0.1 to 0.9). The results are shown in SI, Fig. S3 and indicate that the 1:1 IND:OME ratio is the only one presenting all the new peaks of the new crystalline phase, even though a small contamination of the starting materials is always present. Longer milling times, i.e., above 60 min, will lead to the amorphization of the new crystalline phase together with the small contamination of crystalline IND and OME, precluding the complete formation of the new crystalline phase.

To determine the structure of the new IND:OME crystalline phase, attempts to obtain single crystals from recrystallization in an ethanol solution were performed without success due to OME decomposition. An additional approach for structure solution was further considered by using high resolution PXRD data collected at the synchrotron DESY in Hamburg (Germany) (Fig. 3). The new powder pattern was further indexed using TOPAS and DASH programs (Coelho, 2018, 2017; David et al., 2006; Kabova et al., 2017; Rietveld, 1969, 1967). Several possible unit cells were obtained with non-reasonable volumes and number of molecules per unit cell (Z value), which precluded the correct structure determination (SI, Fig. S5 and S6). From the powder patterns presented in Fig. 3 (where the background was subtracted from the empty capillary) it is possible to observe that some possible residual peaks from IND and OME crystalline phases are still present, which affected the indexing step. Unfortunately, excluding those peaks from the indexing process did again not result in reasonable unit cells and cell volumes for further structure solution by PXRD.

FTIR spectroscopy (Fig. 4a and Fig. S4) and thermal analyses (TGA and DSC) were used to further characterize the new IND:OME crystalline phase. This phase showed new stretching bands at 1590 cm\(^{-1}\) and 1373.5 cm\(^{-1}\), corresponding to C=O asymmetric and symmetric vibrations of a carboxylate functional group. This indicates that a proton transfer occurred from the IND carboxylic group to the OME secondary amine of the imidazolium ring, thus supporting the (predominant) formation of a salt. The TGA curves (Fig. 4b), obtained under oxidative atmosphere (air) conditions, show that the salt is stable up to the onset temperature of ca. 131 °C, presenting a lower thermal stability than the pure crystalline IND (130 °C) and the OME (140 °C). Above this temperature two consecutive steps of mass loss can be observed until 200 °C.

The DSC measurements (Fig. 4c) were carried out under inert atmosphere (N\(_2\)) conditions and in a “semi-closed” environment (perforated lids on the aluminum crucibles). The events show a small shift to higher temperatures when compared to the values obtained from TGA. The salt presented an endothermic peak at 127.5 °C, with a T\(_{\text{onset}}\) at 120.4 °C (Fig. 4c) attributed to its m.p., which can be seen in Fig. 3d at 130 °C, evidencing lower physical stability under heating when compared to crystalline IND (m.p. at 160.8 °C) and OME (m.p. at 163.3 °C). The exothermic peak observed after the melting temperature (Fig. 4c) is attributed to the salt decomposition, as a mass loss is detected in TGA at the same temperature range (i.e., ca. 121.5 °C), defined as an incongruent melt (i.e., melting followed by decomposition).

3.2. Preparation and characterization of the IND:OME co-amorphous system

An IND:OME co-amorphous system was prepared in a 1:1 M ratio using ball mill grinding under dry conditions. The preparation was monitored by PXRD analysis at different milling times. The formation of an amorphous phase is shown in Fig. 5. After 20 min of ball mill grinding under dry conditions, almost full amorphization is achieved and maintained for the 60 min of grinding. The characteristic halo for amorphous materials is observed, indicating that both drugs are amorphized. However, PXRD data alone does not allow us to conclude if a homogeneous co-amorphous system (single amorphous phase with one T\(_g\) value) or a heterogeneous amorphous system (two amorphous phases with two different T\(_g\) values) has been formed.

Further characterization of the IND:OME dry grinded system has been performed using FTIR spectroscopy and thermal analyses (TGA and DSC) (Fig. 6). Interestingly, and in contrast to the salt, the asymmetric C=O stretching band from the carboxylate group of IND did not appear in the IND:OME dry grinded system, which indicates the formation of a neutral (non-charged) system. Additionally, when comparing the FTIR spectra of the isolated amorphous forms of IND and OME with the spectra of IND:OME dry grinded system, some significant changes are noticed, such as the displacement and decrease of the stretching band at 1671 cm\(^{-1}\), corresponding to the C=O of carboxylic group from IND, and the deformation of the N-H group from benzimidazole functional group of OME at 1629 cm\(^{-1}\). This result suggests that hydrogen bonding interactions are formed between these two functional groups of IND and OME molecules in IND:OME dry grinded system.

The TGA data (Fig. 6b) show that the IND:OME dry grinded system has a lower thermal stability than the isolated amorphous forms of IND and OME. A slight initial mass loss of 0.6 % is observed, starting at the beginning of the TGA curve, associated with dehydration of adsorbed water, which usually occurs in amorphous materials. The thermal decomposition of the IND:OME dry grinded system is observed at ca. 90 °C and after this, similar to the salt, a mass loss is detected in two consecutive steps until 200 °C. Only a single T\(_g\) (midpoint at 51.8 °C) is observed in the IND:OME dry grinded system, confirming the formation of a co-amorphous system (see Fig. 6c) with a higher T\(_g\) than the isolated amorphous forms of IND (47.0 °C) and IN (46.2 °C).

3.3. Solid-state phase interconversions and molecular-level elucidation of the IND:OME co-amorphous system

Amorphous forms of IND and OME were used to prepare both IND:OME salt and IND:OME co-amorphous at a 1:1 M ratio. Here the purpose was to evaluate if the solid-state form of the starting materials would affect the outcome during the grinding process, e.g., if by dry grinding amorphous IND and OME, a co-amorphous form would be still formed (pathway ii), as already observed when dry grinding of crystalline IND and OME (pathway i). Furthermore, we also conducted additional experiments to understand if interconversions between IND:OME salt and IND:OME co-amorphous system would occur, e.g., if dry grinding of the salt would lead to the formation of a co-amorphous system. For this,

![Fig. 3. Synchrotron PXRD patterns of IND, physical mixture of IND and OME, and the new IND:OME crystalline phase after LAG for 40 min. Dashed lines in red and blue highlight the possible contamination by crystalline IND and OME, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
with the same thermal profile have been obtained via all tested pathways (i – iii), presenting similar T\(_{\text{\degree C}}\) midpoints: 51.8 \(\pm\) 1.0 \(\degree\) C (i), 51.2 \(\pm\) 1.0 \(\degree\) C (ii), and 51.7 \(\pm\) 1.0 \(\degree\) C (iii), respectively. These results indicate that when using pathway (iii) to produce IND:OME co-amorphous system (i.e., from salt to co-amorphous system), there is a reversing proton exchange (i.e., the proton goes back from OME to IND), thus converting the salt into a neutral co-amorphous system. However, the reverse process (i.e., LAG from co-amorphous phase to salt) could not be realized, since the system showed partial decomposition of OME (colour change to dark purple) (Ronchi et al., 2019).

X-ray total scattering data was collected for IND:OME co-amorphous system, obtained by the three pathways, at the P02.1 beamline, at the DESY synchrotron (Hamburg, Germany). The short wavelength used (0.207 Å), combined with an appropriate data collection strategy, enabled data to be recorded over an acceptable Q-range (in this case, Q\(_{\text{max}}\) = 15 Å\(^{-1}\)) to provide reasonable resolution in real-space for structural analysis. The reduced total scattering structure function, f(q), and the reduced PDF, g(r), were obtained and are presented in Fig. 7a and Fig. S1, respectively. The PDFs were similar for all obtained IND:OME co-amorphous system, regardless of the chosen preparation pathway (Fig. 7), suggesting similar intramolecular and on-average intermolecular distributions.

Molecular dynamics (MD) simulations were used to generate IND:OME co-amorphous system and further obtain the theoretical PDF curve. As can be observed in Fig. 8a, both experimental and simulated PDFs are similar until 7.2 Å, indicating that the generated model is a good representation of the system obtained experimentally. Such good agreement between experimental and simulated PDF data has been previously demonstrated for other amorphous systems (Martins et al., 2023). A comparison between the PDF of the single IND and OME molecules and both simulated and experimental PDFs of IND:OME co-amorphous system (Fig. 8b and c) shows a match between both data from 1.5 Å to 7.2 Å, suggesting that those PDF peaks mostly represent the intramolecular correlations. Furthermore, from 7.2 Å to 13 Å, not only intramolecular correlations are present (the structural dimension of both IND and OME are around 13 Å), but also intermolecular correlations are observed in IND:OME co-amorphous system, which are not easily detected in the experimental PDF due to its resolution limits (especially above 7.2 Å).

To better determine the intermolecular correlations in the IND:OME co-amorphous system, radial distribution functions were calculated. As can be observed in Fig. 9, the COO-H ⋯ O = COH interaction between IND molecules occurs with a higher probability followed by S = O ⋯ H-N (between OME molecules), COO-H ⋯ O = S and HOOC = O ⋯ H-N between IND and OME. These results indicate that both IND-IND and OME-OME interactions are strong and prevalent in IND:OME co-amorphous system. Furthermore, the presence of these interactions can be correlated to the decrease in intensity and displacement of the C=O and N-H vibrational bands from both carboxylic and benzimidazole groups of IND and OME, respectively (Fig. 6a).

### 3.4. Physical stability

The physical stabilities of the amorphous forms of IND and OME, and IND:OME co-amorphous system were evaluated under dry storage conditions at room temperature and the PXRD results are presented in Fig. 10. Conversion of the amorphous forms to crystalline states can be observed by the appearance of Bragg peaks. The isolated amorphous forms of IND and OME prepared by ball mill grinding show very low physical stability even in controlled storage (4 h for IND, and 16 h for OME), converting to the corresponding crystalline polymorphic forms (γ form of IND and A form of OME). The crystallization of amorphous material prepared by ball mill grinding occurs at a faster rate than the material produced by other methods such as quench-cooling or spray dryer (Chiang et al., 2009; Kao et al., 2012), possibly because crystalline seeds of the original polymorph form remain after grinding, which are not detected in the PXRD experiments (Kao et al., 2012). However, no
Fig. 6. (a) FTIR spectra of the amorphous forms (am) of IND and OME, and IND:OME dry grinded system ($\delta$ = deformation; $\nu$ = stretching and BI = benzimidazole). (b) TGA curves and (c) DSC curves of the amorphous forms of IND and OME, and IND:OME dry grinded system.

Fig. 7. (a) PXRD patterns, (b) DSC data, and (c) PDF data of IND:OME co-amorphous systems (prepared via pathways (i) to (iii)).

Fig. 8. (a) Experimental and simulated PDF data of IND:OME co-amorphous system, (b) simulated PDF data of IND, OME and IND:OME co-amorphous system, (c) comparison of simulated PDF of IND and OME with experimental PDF of IND:OME co-amorphous system.
literature has been found for the stability of pure amorphous OME form, except its salts, and amorphous IND produced by other techniques shows crystallization after a few days widely depending on storage conditions (Kao et al., 2012).

Despite the low stability of the amorphous forms of IND and OME, when these drugs are interacting in a homogeneous amorphous phase (IND:OME co-amorphous system), a higher stability of the system is observed and no crystallization became evident for the duration of the stability study (100 days). The chemical stability of IND:OME co-amorphous system was also evaluated during the same time period (i.e., over 100 days) by HPLC and no sign of degradation of OME (Ronchi et al., 2019) was observed (SI, Fig. S7).

3.5. Dissolution studies

The dissolution behaviour of IND in both IND:OME salt and IND:OME co-amorphous system was investigated in phosphate buffer (pH 7.2) and compared with the individual crystalline IND under the same conditions (Fig. 11). The measurements were conducted at a wavelength of 350 nm to avoid potential interference with OME absorption, as illustrated in Fig. 11b. Dissolution rate (DR) calculations are based on the dissolution of IND during the first 10 min of the experiment. The results show a faster dissolution rate for IND from both the co-amorphous system (DR = 36.00 µg/min) and the salt (DR = 21.21 µg/min), when compared to the dissolution of the crystalline IND (DR = 5.33 µg/min). Fig. 11c shows the bar graph representing the area under the dissolution curve (AUC0-8 h) of each sample tested, where the IND:OME salt and co-amorphous system exhibited a 1.8-fold and 2.5-fold higher amount of IND dissolved than for the pure crystalline IND, respectively. The IND concentration at the end of the dissolution test was found to be 31.6 % of the theoretical concentration of fully dissolved drug (for crystalline IND), 58.1 % (for the IND:OME salt) and 78.6 % (for the IND:OME co-amorphous system). Nonetheless, a colour change to dark purple at the end of the assays was observed for the IND:OME co-amorphous system, which is related to OME degradation.

Fig. 9. Radial distribution function data for IND-IND, OME-OME and IND-OME interactions present in the IND:OME co-amorphous system.

Fig. 10. PXRD data of amorphous forms of IND and OME, and the IND:OME co-amorphous system, obtained by ball mill grinding and after storage at room temperature in a desiccator with anhydrous calcium chloride.
This result suggests that the chemical stability of OME when dissolved from a co-amorphous system is lower than when dissolved from a salt.

3.6. In vivo anti-inflammatory activity

The anti-inflammatory activities of both IND:OME salt and IND:OME co-amorphous system were evaluated by an in vivo model of inflammation inhibition using carrageenan-induced mice paw oedema method (Fig. 12). The analyses were performed by measuring the paw before (baseline group) and after oedema induction, and the activity of IND: OME salt and IND:OME co-amorphous system was compared with both positive (IND) and negative (vehicle, without treatment) control groups. The results show that the average paw thickness 1 h after oral administration of the compounds is: 2.30 mm (no treatment), 2.13 mm (IND), 2.01 mm (IND:OME salt) and 1.51 mm (IND:OME co-amorphous system); and after 6 h is: 1.12 mm (no treatment), 0.98 mm (IND), 0.72 mm (IND:OME salt), 0.66 mm (IND:OME co-amorphous system). The percentage of edema inhibition observed at the assay at 6 h is: 13 % (IND), 36 % (IND:OME salt), and 41 % (IND:OME co-amorphous system). Considering the standard deviation values (Fig. 12), there are no statistically significant differences between the values obtained for the IND:OME salt and the co-amorphous system.

Previous studies have demonstrated the efficacy of IND in similar mice models after oral administration (i.e., 10 mg/kg) with an initial stage of anti-inflammatory response within the first 2–3 h (Bruno et al., 2005; Sugishita E, 1981). Our results show a faster and greater response after administering IND:OME in both salt and co-amorphous forms containing half dose of IND (10 mg/kg of the compounds in a 1:1 M ratio, i.e. ~ 5 mg/kg of IND). The enhancement of the pharmacological activity of IND can be explained by the dissolution behavior of both IND:OME salt and IND:OME co-amorphous system (see section 3.5), as higher concentrations of IND are achieved in a shorter time (which may explain the rapid decrease of paw thickness 1 h after administration).

According to the literature, the pharmacokinetic parameters, such as plasma concentration, half-life (T1/2) plasma, area under the curve (AUC), and renal clearance of crystalline IND were observed to be dose-dependent (Lucas, 2016; Sharav and Benoliel, 1999), i.e., the anti-inflammatory activity of IND is higher when its concentration in increases. Interestingly, in our study we observed that the anti-inflammatory activity of IND in the form of a salt and co-amorphous system is higher than crystalline IND when using lower concentrations, i.e., ~5 mg/kg instead of 10 mg/kg of IND.

3.7. Gastroprotective effect of OME as a salt and co-amorphous system

The in vivo gastroprotective effects of IND:OME salt and IND:OME co-amorphous system were investigated by an indomethacin-induced gastric ulcer model in mice and compared with OME (conventional gastric ulcer protective drug). All groups of mice (except the negative control group I) received the same dose of IND (40 mg/kg) as ulcer-inducing agent. Groups II and III received IND separately and groups IV and V received IND with the same dose but in the form of a salt and co-amorphous system by oral gavage. As can be seen in Fig. 13a and b, the IND treatment (positive control group) was able to induce extensive ulcerative lesions in the stomach of the animals. The groups treated with
IND:OME salt and IND:OME co-amorphous system showed significantly smaller lesion areas compared to the positive control (IND) (p < 0.05). On the other hand, there was no significant difference between IND: OME salt and IND:OME co-amorphous system treated groups. Surprisingly, both IND:OME salt co-amorphous showed a higher mucosal protection than the group treated with crystalline OME, which did not demonstrate a significant difference relative to the negative control group (p > 0.05). Representative pictures of the stomachs of each group are shown in the Fig. 13b.

IND has a higher dissolution rate when incorporated in both IND: OME salt and IND:OME co-amorphous system than crystalline IND, which may imply a shorter retention time in the mucosa, resulting in a decrease of side effects in the gastrointestinal tract as a consequence of a non-prolonged contact time (Saeedi et al., 2011). These results imply that oral administration of IND:OME salt and IND:OME co-amorphous system is pharmacologically favorable compared to taking the individual drugs.

4. Conclusions

IND:OME salt was obtained after 40 min of LAG in the presence of a small amount of ethanol (60 μL). Small impurities from crystalline starting materials have been detected in the salt by PXRD. Attempts to solve the crystal structure of IND:OME salt have failed, even when excluding the diffraction peaks coming from both crystalline IND and OME. FTIR results showed a proton transfer from the IND carboxylic group to the OME secondary amine of the benzimidazolium group, thus confirming the salt formation. Ball mill grinding under dry conditions of IND with OME led to a complete amorphization after 60 min, forming a homogeneous co-amorphous system according to the DSC (single Tg at 51.8 °C) and a neutral compound according to the FTIR data (no proton transfer from the IND carboxylic group to the OME secondary amine). Surprisingly, IND:OME salt converted into a neutral co-amorphous system when subjected to ball mill grinding under dry conditions (Tg of 51.7 °C, but the reverse process (LAG of IND:OME co-amorphous system in ethanol) showed a partial chemical decomposition of OME. IND:OME co-amorphous system presented higher physical stability (longer than 100 days) than the amorphous forms of IND (4 h) and OME (16 h). OME presented higher chemical stability in solution when integrated as a salt (IND:OME). The dissolution study showed a faster dissolution rate at 10 min for IND in the co-amorphous (Dh = 36.00 μg/min) and salt (Dh = 21.21 μg/min), when compared with the crystalline IND (Dh = 5.39 μg/min). In addition, the AUC calculations demonstrate a dissolution enhancement for IND in both IND:OME salt (1.8-fold) and IND:OME co-amorphous system (2.5-fold) after 8 h. In vivo anti-inflammatory activity of IND showed a faster and greater response when administered in the form of a salt (3.0-fold) and co-amorphous system (3.1-fold), at half a dose (~5 mg/kg). Moreover, IND:OME salt and IND:OME co-amorphous system presented an unexpected higher gastroprotective performance in vivo than crystalline OME as a result of the improvement in their physicochemical properties.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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