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Microwave assisted synthesis of new 2-aryl and 2-alkylimidazolones and evaluation of their in vitro anticancer activity and their in vivo toxicity on zebrafish embryos

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Abstract

Despite the development of many treatment strategies, cancer is still considered as a major cause of the death worldwide. It is therefore of great importance to elaborate more effective and targeted treatment therapies. Several molecules of marine natural sources bearing a 2-aminoimidazolone scaffold, such as leucettamine B and its synthetic analogs, have revealed interesting spectrum of biological activity. For instance, some 2-aryl or 2-alkylimidazolones have also been synthesized; however their anticancer activity was less explored. Herein we describe the synthesis of five new 2-aryl and 2-alkylimidazolones derivatives via an effective one-pot synthetic strategy assisted by microwave irradiations which allowed us to access the product **2aa** in a reduced time reaction compared to the thermal heating, with slightly better yield (48% compared to 45%). The new imidazolones derivatives were evaluated for their potential anticancer activity *in vitro* against MCF-7, MDA-MB-231 and HepG2 cell lines. Some of these derivatives exhibited interesting cytotoxic effects on both MCF-7 and HepG2 cell lines showing that this family of compounds could be considered as potential anticancer agents. Furthermore, their toxic effect was evaluated *in vivo* on the development of zebrafish embryos. The latters exhibited some morphological abnormalities when treated with high concentrations of the synthesized compounds.

Keywords: Imidazolones derivatives, One-pot synthesis, Microwave irradiations, Anticancer activity, Zebrafish embryos.

Introduction

Cancer is a major public health problem worldwide; it is considered the second leading cause of death globally (Wild et al. 2020). In particular, breast cancer is rated as the second most commonly diagnosed cancer and the leading cause of cancer death among females (DeSantis et al. 2017). Hepatocellular carcinoma (HCC) is considered the sixth most frequently diagnosed cancer and the fourth leading cause of death from cancer in the world (Bray et al. 2018). Breast tumors are very heterogeneous and can be classified as tumors expressing estrogen and progesterone receptors (ER+, PR+), tumors expressing amplified human epidermal receptors (HER2+) and tumors not expressing these receptors, called triple negative breast (ER-, PR-, HER2-) (Dai et al. 2017). As for the HCC, studies have shown an overexpression of HER2 protein in several HCC cell lines (Shi et al. 2019). Considerable efforts

59 have been invested in establishing efficient treatment strategies of which the most commonly
 60 used are surgery, radiotherapy and drug treatments (You et al. 2007; Luqmani 2005). In spite
 61 of being effective in the treatment of several types of cancer, especially in the case of primary
 62 tumors, these strategies can induce tumor heterogeneity resulting in the reduction of the
 63 therapeutic agents efficiency and leading to treatment failure and tumor progression (Foo and
 64 Michor 2014; Luqmani 2005). Thus, it is necessary to develop new targeted therapies aiming
 65 specific cellular mechanisms. Many natural products classified as leads to potential drugs,
 66 contribute significantly in the drug discovery and development process especially in cancer
 67 treatment (Newman and Cragg 2012). As a matter of fact, marine natural products isolated from
 68 marine sponges and organisms, with 1490 new products extracted and identified in 2017
 69 (R. Carroll et al. 2019), are being explored for their different medical applications (Mayer et al.
 70 2011; Villa and Gerwick 2010). Among these natural marine products, several molecules based
 71 on a 2-aminoimidazolone core, such as leucettamine B (Boehm et al. 1993; Chan et al. 1993),
 72 have shown interesting biological properties. Leucettamine B (**Erreur ! Source du renvoi**
 73 **introuvable.**) and its synthetic N-functionalized analogs have manifested significant inhibitory
 74 activities against protein kinases, CLK1 (CDC (Cyclin-dependent kinase)-like kinase), and
 75 DYRKs (Dual-specificity tyrosine phosphorylation-regulated kinases) (Burgy et al. 2013;
 76 Tahtouh et al. 2012; Debdab et al. 2010, 2011). In the last years the imidazolone scaffold have
 77 attracted attention as a potential block for the development of new effective anticancer agents
 78 (Hsu et al. 2020; Abdel Gawad et al. 2016; Ling et al. 2013; Xiao et al. 2013). Moreover,
 79 analogs of type 2-aryl or 2-alkylimidazolones (**Erreur ! Source du renvoi introuvable.**) have
 80 also been synthesized (Mokale et al. 2014; Voosala et al. 2014; Shi et al. 2012; Fozooni and
 81 Tikdari 2008); however, the evaluation for their anticancer activity is limited.

82
 83 **Fig. 1** Structure of leucettamine B and targeted 2-aryl and 2-alkylimidazolones

84 In fact, some synthesized (Z)-2-aryl-5-arylmethylideneimidazolones were reported to
 85 have potent cytotoxic effects against epithelial carcinoma cancer cell line A549 (Beloglazkina
 86 et al. 2016). Eldehna et al. (Eldehna et al. 2019) also reported the synthesis and biological
 87 evaluation of novel 4-benzylidene-2-phenyl-imidazolone-based benzenesulfonamides that

88 showed anti-proliferative activity against colorectal cancer HCT-116 and breast cancer MCF-7
 89 cell lines. The synthetic methodologies adopted to access these imidazolones derivatives have
 90 been established on the transformation of oxazolone nuclei into imidazolone unit according to
 91 several operating conditions. An efficient multi-component strategy was developed by Kidawi
 92 et al. (Kidwai and Devasia 1962), involving the reaction of the ethyl ester of glycine with
 93 imidate esters and aromatic aldehydes. Nevertheless, it was less adopted due to the tedious
 94 preparation of imidate salts that required the passage of a continuous flow of hydrogen chloride
 95 gas over a solution of the nitriles and alcohol in ether or benzene. A new method for
 96 synthesizing these imidate salts by generating hydrogen chloride *in situ* (Yadav and Babu
 97 2005), made the Kidawi's one-pot approach much more interesting. In light of the above
 98 evidence and inspired by the biological activity of imidazolones derivatives, an effective one-
 99 pot microwave assisted synthesis was applied to access a library of new 2-aryl and 2-
 100 alkylimidazolones derivatives as potential anticancer agents (Scheme 1). This non-conventional
 101 heating has emerged as an efficient green method to enhance products yield and to reduce
 102 reaction time (Kappe and Dallinger 2009; Strauss and Varma 2006).

103

104 **Scheme 1** Microwave-assisted synthetic strategy to imidazolones derivatives

105 The potential anticancer activity of the new compounds was then evaluated *in vitro* on the breast
 106 cancer cell lines MCF-7, MDA-MB-231 and HCC cell line HepG2. Furthermore, we evaluated
 107 their toxic effect *in vivo* using the zebrafish model, considered as a versatile animal model due
 108 to the simplicity of its natural habitat, low cost and ease of breeding (Newman et al. 2014). The
 109 ex-utero development of its transparent embryos, allowed a clear visualization of the
 110 development process (Meyers 2018; Link and Megason 2008) and the evaluation of the
 111 toxicological effects of the newly synthesized imidazolones on this process.

112

113

Experimental

114

Chemistry

116

Chemicals, reagents and instruments

117

118

119 Solvents and all other chemical reagents were purchased from Acros, Sigma Aldrich
120 and Fluka and were used without further purification. Solvents were dried by refluxing with the
121 appropriate drying agents and distilled before use. Toluene was distilled over sodium and stored
122 over molecular sieves (3 Å) and ethanol was distilled over calcium hydride (CaH₂). Melting
123 points were measured on a melting point apparatus Stuart SMP10 and were not corrected. ¹H
124 NMR spectra were recorded on BRUKER AC 300 and AC 400 (300 and 400 MHz) and ¹³C
125 NMR spectra on BRUKER AC 300 and AC 400 (75 and 101 MHz) spectrometer. Chemical
126 shifts are expressed in parts per million downfield from tetramethylsilane as an internal
127 standard. Data are given in the following order: δ value, multiplicity (s: singlet; d: doublet; t:
128 triplet; q: quartet; m: multiplet; br: broad), number of protons, coupling constant *J* is given in
129 Hertz. All high-resolution mass spectra (HRMS) were recorded on a Bruker Micro-Tof-Q II or
130 on a Waters Q-Tof 2 at the CRMPO (Centre Régional de Mesures de Physiques de l'Ouest–
131 Rennes-France) using positive ion electrospray. Reactions under microwave irradiations were
132 realized in the apparatus Anton Paar's Monowave® 300 (ScanMat Platform, Rennes, France).
133 The microwave instrument consists of a continuous focused microwave power output from 0
134 to 850 W. All experiments were performed using stirring option at 600 rpm. The target
135 temperature was reached within the required time and the chosen microwave maximal power
136 was set to 100 W. The reaction temperature is monitored using calibrated infrared sensor and
137 the reaction time. The imidates salts **1a** (Kumar et al. 2016), **1b** (Yadav and Babu 2005), **1c**
138 (Yadav and Babu 2005) and **1d** (Shi et al. 2018) were prepared as previously described.

139 *General procedure for the synthesis of 5-benzylidene-2-aryl/2-alkyl-3,5-dihydro-4H-*
140 *imidazol-4-one 2*

141

142 Glycine methylester hydrochloride (1.5 eq), the corresponding acetimidate
143 hydrochloride (1.1 eq) and sodium bicarbonate (3 eq) were poured in the microwave vial,
144 flushed with argon, and anhydrous toluene (0.8 mL) was added to the powders. To the
145 suspension was then added the aldehyde (1 eq), the vial was then placed in the Anton Paar
146 monowave reactor and the reaction was stirred during 15 minutes at 150 °C and at 100 W of
147 maximum power. After the reaction completion, the suspension was allowed to precipitate at
148 room temperature. The yellow solid obtained was collected by filtration, washed twice with
149 water, twice with cold ethanol and twice with isopropyl ether to afford the corresponding
150 imidazolone as a yellow powder.

151 (Z)-5-(4-methoxybenzylidene)-2-phenethyl-3,5-dihydro-4H-imidazol-4-one **2aa**

152 General procedure starting from *p*-methoxybenzaldehyde (115 mg, 0.85 mmol) and **1a** (200
153 mg, 0.936 mmol). Yield, 48%; yellow solid, mp 181-183 °C. ¹H NMR (400 MHz, DMSO, δ
154 ppm): 8.16 (d, *J* = 8.9 Hz, 2H), 7.30 (d, *J* = 4.4 Hz, 4H), 7.23-7.17 (m, 1H), 7.02 (d, *J* = 8.9 Hz,
155 2H), 6.83 (s, 1H), 3.82 (s, 3H), 3.04 (t, *J* = 7.8 Hz, 2H), 2.84 (t, *J* = 7.8 Hz, 2H). ¹³C NMR (101
156 MHz, DMSO, δ ppm): 172.1, 165.2, 161.1, 141.1, 138.6, 134.1, 128.8, 128.8, 127.4, 126.6,
157 124.5, 114.7, 55.8, 31.9, 31.6. HRMS (ESI⁺): *m/z* calcd for C₁₉H₁₈N₂O₂Na [M+Na]⁺: 329.1260,
158 found 329.1259.

159 (Z)-5-(3-methoxybenzylidene)-2-phenethyl-3,5-dihydro-4H-imidazol-4-one **2ab**

160 General procedure starting from *m*-methoxybenzaldehyde (115 mg, 0.85 mmol) and **1a** (200
161 mg, 0.936 mmol). Yield, 26%; yellow solid, mp 172-174 °C. ¹H NMR (300 MHz, DMSO, δ
162 ppm): 7.89 (s, 1H), 7.71 (d, *J* = 7.7 Hz, 1H), 7.38-7.27 (m, 5H), 7.24-7.15 (m, 1H), 6.97 (dd, *J*
163 = 7.9, 2.7 Hz, 1H), 6.82 (s, 1H), 3.78 (s, 3H), 3.04 (t, *J* = 7.7 Hz, 2H), 2.85 (t, *J* = 7.7 Hz, 2H).
164 ¹³C NMR (101 MHz, DMSO, δ ppm): 172.3, 167.1, 159.7, 141.1, 140.7, 135.9, 130.0, 128.8,
165 128.8, 126.6, 124.9, 124.0, 117.0, 116.1, 55.5, 31.8, 31.3. HRMS (ESI⁺): *m/z* calcd for
166 C₁₉H₁₈N₂O₂Na [M+Na]⁺: 329.1260, found 329.1264.

167 (Z)-5-(4-methoxybenzylidene)-2-(4-methoxyphenyl)-3,5-dihydro-4H-imidazol-4-one **2b**

168 General procedure starting from *p*-methoxybenzaldehyde (114 mg, 0.84 mmol) and **1b** (200
169 mg, 0.927 mmol). Yield, 70%; yellow solid, mp 262-264 °C. ¹H NMR (300 MHz, DMSO, δ
170 ppm): 8.28 (d, *J* = 8.9 Hz, 2H), 8.12 (d, *J* = 8.9 Hz, 2H), 7.14 (d, *J* = 8.9 Hz, 2H), 7.06 (d, *J* =
171 8.8 Hz, 2H), 6.91 (s, 1H), 3.87 (s, 3H), 3.84 (s, 3H). ¹³C NMR (101 MHz, DMSO, δ ppm):
172 172.6, 163.0, 161.0, 159.7, 139.3, 134.2, 129.6, 127.8, 124.3, 120.9, 114.9, 114.8, 56.0, 55.8.
173 HRMS (ESI⁺): *m/z* calcd for C₁₈H₁₆N₂O₃Na [M+Na]⁺: 331.1053, found 331.1056.

174 (Z)-2-(4-chlorophenyl)-5-(4-methoxybenzylidene)-3,5-dihydro-4H-imidazol-4-one **2c**

175 General procedure starting from *p*-methoxybenzaldehyde (112 mg, 0.826 mmol) and **1c** (200
176 mg, 0.91 mmol). Yield, 64%; yellow solid, mp 272-274 °C. ¹H NMR (300 MHz, DMSO, δ
177 ppm): 8.29 (d, *J* = 8.8 Hz, 2H), 8.16 (d, *J* = 8.6 Hz, 2H), 7.67 (d, *J* = 8.6 Hz, 2H), 7.07 (d, *J* =
178 8.8 Hz, 2H), 7.01 (s, 1H), 3.84 (s, 3H). ¹³C NMR (75 MHz, DMSO, δ ppm): 172.4, 161.4,
179 159.2, 138.9, 137.4, 134.6, 129.6, 129.4, 127.6, 127.6, 126.2, 114.9, 55.8. HRMS (ESI⁺): *m/z*
180 calcd for C₁₇H₁₃N₂O₂ClNa [M+Na]⁺: 335.0557, found 335.0556.

181 (Z)-2-(4-bromophenyl)-5-(4-methoxybenzylidene)-3,5-dihydro-4H-imidazol-4-one **2d**

General procedure starting from *p*-methoxybenzaldehyde (93.5 mg, 0.687 mmol) and **1d** (200 mg, 0.756 mmol), Yield, 79%; yellow solid mp 286-290 °C. ¹H NMR (300 MHz, DMSO, δ ppm): 8.29 (d, *J* = 8.8 Hz, 2H), 8.09 (d, *J* = 8.6 Hz, 2H), 7.81 (d, *J* = 8.6 Hz, 2H), 7.07 (d, *J* = 8.9 Hz, 2H), 7.03 (s, 1H), 3.84 (s, 3H). ¹³C NMR (75 MHz, DMSO, δ ppm): 172.3, 161.4, 159.2, 138.9, 134.5, 132.5, 129.5, 127.8, 127.5, 126.4, 126.2, 114.9, 55.8. HRMS (ESI+): *m/z* calcd for C₁₇H₁₃N₂O₂BrNa [M+Na]⁺: 379.0052, found 379.0050.

188

189 **Biological activity**

190 *Cell lines: Culture conditions and viability assays*

191

MCF-7, hormone-dependent human breast cancer, MDA-MB-231, hormone-independent human breast cancer and HepG2 hepatocellular carcinoma cell lines were purchased originally from ATCC (American Type Culture Collection). MCF-7 and MDA-MB-231 cells were routinely maintained in RPMI 1640 medium (Sigma R0883) and HepG2 in DMEM high glucose medium (Sigma D0819), both medium containing 1% penicillin streptomycin (Biowest MS006W) and supplemented with 10% Fetal Bovine Serum (Sigma F9665) and 1% L-Glutamine (Sigma G7513). The cells were cultured at 37 °C in a humidified incubator with 5% CO₂ and were passaged twice a week using 1% trypsin (Sigma T4549). The cytotoxic effects of the imidazolones derivatives were determined using the trypan blue exclusion assay. It is based on the concept of dye exclusion to differentiate viable cells from dead ones. Indeed, cells that possess damaged membranes are stained blue whereas viable cells do not uptake the dye and remain white. MCF-7, MDA-MB-231 and HepG2 cells were seeded into 24-well culture plates (20,000/well), in a total volume of 500 μL/well. After overnight incubation to ensure cell attachment and well-being, the medium was aspirated and cells were exposed to the synthesized imidazolones in duplicate at final concentrations ranging from 0.1 to 60 μM. Stock solutions of these compounds at 30 mM were prepared in DMSO (Sigma-Aldrich 41640) and then diluted in culture medium to obtain the desired final concentrations. The highest concentration of DMSO following dilution was 0.2% of the total volume, an amount that was not toxic to the cells. Control cells were grown in culture medium. The vehicle cells were treated with 0.2% of DMSO. Plates were incubated for 24, 48, and 72 hours. At the end of each time point, the medium was removed; the cells were collected by the action of trypsin and counted in a hemocytometer using optical microscopy. The results were presented

214 as a percentage of the control. All measurements were performed in duplicate and each
215 experiment was repeated at least three times independently.

216

217 *Animals*

218

219 Adult wild-type zebrafish (*Danio rerio*) of AB and Casper strain of both sexes were
220 purchased from UMS AMAGEN CNRS INRA (France) and were used after ethical approval.
221 Animals were divided in 5 L thermostated tanks at 28 ± 2 °C (15 fish/tank) in a constant
222 chemical and biological controlled environment according to international standards.
223 Mechanical water filtration and aeration were also kept under constant control. Animals were
224 maintained under a 14-10 hours day/night photoperiod cycle and fed three times a day with
225 commercial flakes and live brine shrimp. Natural spawning was induced in the morning by
226 light. Embryos were then collected within 30 min and maintained in an E3 (1X) medium in an
227 incubator at 25 °C to minimize stress and spontaneous deaths when treating.

228 E3 medium (60X) is the adequate medium for zebrafish embryos and was prepared as
229 followed: 34.8 g sodium chloride, 1.6 g potassium chloride, 5.8 g calcium chloride dihydrate,
230 9.78 g magnesium chloride hexahydrate were dissolved in water with a final volume 2 L to
231 prepare a 60X stock. This solution was autoclaved before adjusting the pH and diluting, after
232 which it becomes compatible to use for zebrafish embryos.

233

234 *Zebrafish embryo toxicity assay: Exposure to the imidazolones derivatives*

235

236 Fertilized eggs of zebrafish between late blastula to early gastrula (2.5-5.25 hpf (hours
237 post-fertilization)) were sorted to ensure only eggs of the same quality were used in the
238 experiments. This was done to minimize spontaneous defects and variability between different
239 conditions. Zebrafish embryos were then transferred to 24 well plates (5 to 10 embryos per
240 well) and were maintained in 0.5 to 1 mL of E3 medium. Embryos were then exposed to
241 different concentrations of imidazolone derivatives (1 μ M to 120 μ M). Stock solutions of these
242 compounds at 30 mM were prepared in DMSO and then diluted in E3 medium to obtain the
243 desired final concentrations. The highest concentration of DMSO following dilution was 0.5%
244 of the total volume. A control group (E3 1X) and a vehicle group (0.5% DMSO) were part of
245 the experiment. Fish embryonic development was observed directly using a stereoscope, and
246 pictures were taken using the AmScope software. The malformations observed and the viability
247 rates were noted and calculated after 24, 48 and 72 hours of treatment.

Results and discussion

Chemistry

The imidate salts used in this approach were synthesized according to the protocol described by Veejendra Yadav et al. (2005). This method is based on the generation of hydrogen chloride *in situ* following the treatment of acyl chloride with an alcohol (Scheme 2).

Scheme 2 Preparation of imidate salts

The imidate salts were subsequently used in the preparation of 2-aryl and 2-alkylimidazolones according to the strategy described first in 1962 (Kidwai and Devasia 1962), while making modifications to the reaction conditions. It is a three-component one-pot reaction involving an imidate salt, a glycine ester and an aromatic aldehyde (Scheme 3). At this stage, two methods of activation of the reaction have been employed. The process was carried out under conventional thermal conditions and also under microwave irradiation in order to compare the two activation modes. In the interest of optimizing the conditions, the reaction was conducted using *p*-methoxybenzaldehyde and compound **1a**, chosen as model compounds, in the presence of sodium hydrogen carbonate as base. In conventional thermal conditions (toluene, 70 °C), the product was isolated with 45% yield (in 97% of conversion) after 2 hours of reaction whereas the same reaction conducted in the same conditions without solvent did not result in the desired product but rather a degradation of the starting products was observed. Furthermore, the reaction was conducted under microwave irradiations and the conditions were optimized by variation of the heating temperature and the reaction time. In a first attempt, the reaction was carried at 100 °C under 100 W of maximum power and for 15 min to yield the desired product in 88% of conversion. In the next assay the temperature was increased to 150 °C which resulted in a better conversion (99.7%) and better yield (48%) of isolated product

275 compared to thermal heating. Finally in an attempt to achieve a greener process, the reaction
 276 was conducted in a solvent-free medium in the same conditions as before; however, a decrease
 277 in the conversion to 87% was noted. An increase in the heating temperature to 200 °C in a
 278 solvent-free medium caused a degradation of the imidate salt and did not yield the desired
 279 product. Therefore, in a final attempt, in a solvent-free medium, the temperature was maintained
 280 at 150 °C and the reaction was carried for 30 min. In these conditions the product was obtained
 281 in 92% of conversion that was still lower than the conversion obtained in the presence of
 282 solvent. The microwave assisted method in a solvent-based medium has proved to be more
 283 efficient and suitable to reduce the reaction time and slightly enhance the yield.

284

285 **Scheme 3** Optimization of the one-pot process under microwave conditions, reaction
 286 Conditions: (A) 72 °C 2 hours, (B) 150 °C, 100 W, 15 min

287 Subsequently, the one-pot approach was generalized to several substrates by varying
 288 either the aldehyde or the salt of imidates. The reactions were carried out in the microwave
 289 under the same conditions as above (150 °C, 100 W, 15 min) leading to a small library of
 290 imidazolone derivatives (Fig. 2). The desired products precipitated in the toluene and were
 291 purified by a simple filtration followed by several washes in water and ether. As they were
 292 insoluble in several organic solvents, it was not possible to purify them by column
 293 chromatography. All compounds were obtained in a stereospecific way with retention of
 294 stereochemistry and the geometry of the exocyclic double bond that was attributed as being *Z*

295 by the shielding effect of the carbonyl C-4 on the olefinic proton H-5 ($\delta_{\text{H-5}} = 6.82\text{--}7.05$ ppm)
 296 (Debdab et al. 2010; Villemin and Martin 1995).

297

298

299 **Fig. 2** Yields of the 2-aryl and 2-alkylimidazolones synthesized via one-pot approach; α :
 300 conversion percentage

301 The results showed that the one-pot reaction gives better yields by using imidate salts
 302 of aryl type (**2b**, **2c**, **2d**) compared to those of alkyl type (**2aa**, **2ab**). This observation can be
 303 explained by the stability of the imidazolones formed from the aryl imidate salts, due to the
 304 conjugation of the cyclic double bond with the aromatic nucleus of these salts (Kidwai and
 305 Devasia 1962). It could also be explained by the fact that a small quantity of **2aa** and **2ab**
 306 products was lost in the precipitation purification and was observed in the filtrate after filtration
 307 which was not the case of compounds **2b**, **2c** and **2d**. However, the difference in reactivity
 308 observed within these imidates of aryl type seems to be linked to the nature of the substituents
 309 on the aromatic ring. An electron-donor group on the aromatic nucleus promoted the
 310 intramolecular cyclization of the intermediate, obtained following the addition of glycine to the
 311 imidate salt, by activating the attack of the amine on the ester based on the proposed mechanism
 312 in the online resource. A significant decrease in yield was noticed by variation of the aldehyde
 313 (**2aa** compared to **2ab**). This decrease can be attributed to the difference in stability of the final

314 products obtained. Indeed, *p*-methoxybenzaldehyde induces a stability of the final product **2aa**
315 via the conjugation of the exocyclic double bond with the aromatic nucleus, reinforced by the
316 presence of the methoxy group in the para position. This effect is absent in the case of a methoxy
317 group in the meta position (**2ab**). Furthermore, the absence of an activating group on the
318 aromatic nucleus of the aldehyde did not lead to total conversion. Benzaldehyde, used with **1a**
319 under the same conditions as before, resulted in a 78% conversion to the final product.
320 However, attempts to purify this derivative have not been successful.

321

322 *Cell viability*

323

324 The anticancer effect of the various synthesized imidazolone derivatives was assessed
325 by means of the cell exclusion test with trypan blue against human breast cancer cell lines MCF-
326 7 (Fig. 3) and MDA-MB-231 (Fig. 4) and against HCC cell lines HepG2 (Fig. 5). Doxorubicin
327 was used as positive control. The IC₅₀ (concentration of the compound which caused 50% of
328 cell death) for each cell line were determined based on dose-response curves, concentrations as
329 a function of the viability percentages obtained 72 hours post-treatment. The results are
330 summarized in Table 1. It should be noted that the increased lipophilicity of compounds **2c** and
331 **2d** (log P values of 3.13-3.4) caused problems in assessing their cytotoxic effect. These
332 compounds started to precipitate in the culture medium even at low concentrations (10 µM);
333 therefore it was not possible to determine the exact IC₅₀ values.

334 On breast cancer cell lines, the 2-alkyl and 2-arylimidazolones derivatives **2aa**, **2ab** and
335 **2b** (log P values of 2.44-2.93) were found to be inactive against the proliferation of the
336 hormone-independent breast cancer line MDA-MB-231. As for the hormone-dependent breast
337 cancer line MCF-7, the results show that the highest anticancer activity was observed for the 2-
338 arylimidazolone compound **2b** (IC₅₀ = 7.73 µM) having a methoxy group in the para position
339 on each of the aromatic nuclei. As for the 2-phenethylimidazolone derivatives, compound **2aa**,
340 having a methoxy substitution in the para position of the benzylidene moiety, did not show any
341 interesting cytotoxic effect (IC₅₀ > 30 µM).

342

343 **Fig. 3** Percentage of cell viability of the MCF-7 cell line compared to the control following
 344 treatment with the different concentrations of compounds 2aa (a), 2ab (b), 2b (c), 2c (d) and 2d
 345 (e) after 24, 48 and 72 hours. Each experiment was carried out in duplicate. *, **, ***, ****
 346 indicate a significant difference compared to the vehicle with $p < 0.05$, $p < 0.01$, $p < 0.001$ and p
 347 < 0.0001 respectively according to the ANOVA test

348 However, an enhanced cytotoxic activity was noted, when the methoxy group was displaced
 349 from para to meta position in compound **2ab**, showing an IC_{50} of 11 μM .

350 **Table 1** IC_{50} values of compounds against different cancer cell lines

IC_{50}^* (μM)			
Product	MDA-MB-231	MCF-7	HepG2
2aa	> 60	> 30	12.16
2ab	> 30	11	6.8
2b	26	7.73	9.47
2c	> 10	> 10	> 10
2d	> 10	> 10	> 10
Doxorubicin	0.01	0.022	0.046

351 * IC_{50} values were derived from single dose-response curves generated from duplicate data points obtained after 72 hours of
 352 treatment.

353

354

355 **Fig. 4** Percentage of cell viability of the MDA-MB-231 cell line compared to the control
 356 following treatment with the different concentrations of compounds **2aa** (a), **2ab** (b), **2b** (c), **2c**
 357 (d) and **2d** (e) after 24, 48 and 72 hours. Each experiment was carried out in duplicate. *, **,
 358 ***, **** indicate a significant difference compared to the vehicle with $p < 0.05$, $p < 0.01$, p
 359 < 0.001 and $p < 0.0001$ respectively according to the ANOVA test.

360 When tested on the hepatocellular carcinoma cell line HepG2, the 2-
 361 phenethylimidazolone derivative **2ab** was found possessing the most cytotoxic activity with an
 362 IC_{50} of 6.8 μM. The displacement of the methoxy substitution from the meta position (**2ab**) to
 363 the para position (**2aa**), reduced the cytotoxic activity by half. The 2-arylimidazolone
 364 compound **2b** also showed an interesting anticancer effect against this cell line with an IC_{50} of
 365 9.47 μM. Overall, the 5-benzylidene-2-alkyl and 2-arylimidazolones showed noticeable
 366 cytotoxicity against MCF-7 and HepG2 cell lines. In literature, some 5-benzylidene-2-
 367 arylimidazolones derivatives have also shown anticancer activity against epithelial carcinoma
 368 cancer cell line A549 with IC_{50} values of 0.44 to 12 μM (Beloglazkina et al. 2016).

369

370 **Fig. 5** Percentage of cell viability of the HepG2 cell line compared to the control following
 371 treatment with the different concentrations of compounds **2aa** (a), **2ab** (b), **2b** (c), **2c** (d) and
 372 **2d** (e) after 24, 48 and 72 hours. Each experiment was carried out in duplicate. *, **, ***, ****
 373 indicate a significant difference compared to the vehicle with $p < 0.05$, $p < 0.01$, $p < 0.001$ and p
 374 < 0.0001 respectively according to the ANOVA test

375 The difference of anticancer activity noted between ER+ cell line MCF-7 and HER2+ cell line
 376 HepG2 on one hand and triple negative cell line MDA-MB-231 on the other hand could be
 377 attributed to the different kinases expressions in these cell lines. In fact, it has been reported
 378 that CLK2 is amplified and overexpressed in HER2+ and ER+ cell lines and that
 379 downregulation of CLK2 inhibited breast cancer growth in cell culture (Yoshida et al. 2015),
 380 whereas inhibition of hyperactive CDKs in triple negative breast cancer resulted in decreased
 381 cell migration and invasion (Tarasewicz et al. 2014). These findings could suggest possible
 382 selective interactions of the synthesized imidazolones derivatives with specific kinases.
 383 All the imidazolones derivatives were less potent than the doxorubicin drug used as positive
 384 control. However, the latter can induce acute and chronic cardiotoxicity that may occur at low
 385 doses (Octavia et al. 2012). It also showed toxicity on cardiogenesis, heart formation and
 386 survival of zebrafish embryos (Ma et al. 2018; Chang et al. 2014).

387

388 *Zebrafish embryo toxicity assay*

389

The newly synthesized imidazolones derivatives and doxorubicin drug have been tested for their toxic effect *in vivo* on the development of zebrafish embryos exposed to increasing concentrations (1-120 μ M) of these compounds. Morphological abnormalities and mortality of the embryos were studied. The summary of mortality and observed malformations of zebrafish embryos at 72 hpf with the different compounds are presented in Table 2.

Table 2 Percentage of mortality and types of morphological malformation caused by each analog in zebrafish embryos at 72 hpf

Product	% of mortality (at 72 hpf)	Morphological malformations at 72 hpf			
		Cardiac edema	Yolk sac edema	Curved tail /Short tail	Hatching retardation
2aa					
1	0	-	-	-	-
3	0	-	-	-	-
10	6	-	-	+	-
30	10	+	+	+	-
60	12	+	+	+	-
90	50	-	+	+	+
120	50	-	+	+	+
2ab					
1	0	-	-	-	-
3	0	-	-	-	-
10	5	-	-	-	-
30	5	-	-	-	-
60	13	+	+	+	-
90	15	-	+	+	-
120	13	-	+	+	-
2b					
1	0	-	-	-	-
3	0	-	-	-	-
10	0	-	-	-	-
30	10	-	-	-	-
60	10	-	+	+	-
90	15	+	+	+	-
120	15	+	+	+	-
2d					
1	0	-	-	-	-
3	0	-	-	-	-

10	0	-	-	-	-
2c					
1	0	-	-	-	-
3	0	-	-	-	-
10	0	-	-	-	-
Doxorubicin					
1	0	-	-	-	-
3	0	-	-	-	-
10	20	-	-	-	-
30	50	+	-	+	+
60	100	+	-	+	+
90	100	+	-	+	+
120	100	+	-	+	+

397 *% of mortality (at 72 hpf) for control and vehicle (0.5% of DMSO) are zero and none morphological malformations at 72 hpf
 398 are detectable

399 Below concentrations of 10 μ M, no incidence of mortality and malformation was observed with
 400 all the tested compounds. The mortality rate slightly increased after exposure to **2ab** and **2b** at
 401 concentrations higher than 60 μ M. Furthermore, treatment with **2aa** starting from 60 μ M caused
 402 a significantly increased mortality at 48 and 72 hpf. At 72-96 hpf, there were significant
 403 differences in the malformation phenotypes in the zebrafish embryos that were exposed to the
 404 various compounds. The predominant effects observed after **2ab** (Fig. 6) and **2aa** (Fig. 7)
 405 exposure were pericardial edema, yolk sac edema, short and contorted tail. For **2ab**, starting
 406 from 30 μ M and at 72 hpf, the length of the embryos became much shorter, whereas cardiac
 407 edema, yolk sac edema and short yolk sac extension were well noted at 60 μ M. In addition, a
 408 significant contraction of the tail has been observed at 60 μ M (Fig. 6). However, **2aa** compound
 409 treatment induced tail contraction at 10 μ M and yolk sac and pericardial edema starting from
 410 30 μ M (Fig. 7). Furthermore, a delay in zebrafish hatching was observed only for **2aa**
 411 compound at high concentrations.

412 It was not possible to determine the effect of **2c** and **2d** compounds at high
 413 concentrations due to their precipitation in the E3 medium. After 72 hours of exposure to **2c**
 414 and **2d**, there were no significant mortality, neither effects on hatching delay nor malformation
 415 in embryonic development, when compared with control group with all concentrations used
 416 below 10 μ M. Overall, the 5-benzylidene-2-alkyl and 2-arylimidazolones induced significant
 417 mortality and abnormalities only at high concentrations. These compounds were less toxic on
 418 the development of zebrafish embryos than doxorubin drug that caused 50% of lethality at 30
 419 μ M.

420

421 **Fig. 6** Zebrafish embryos treated with the **2ab** product for 72 hours a) CTRL- Control; b)
 422 embryo treated with 3 μ M c) embryo treated with 30 μ M; d) embryo treated with 60 μ M; e)
 423 embryo treated with 60 μ M; f) embryo treated with 120 μ M. Scale bar 250 μ m. ce: cardiac
 424 edema; yse: yolk sac edema; sy: short yolk sac extension; ct: contracted tail

425

426

427 **Fig. 7** Photos of encountered malformations, resulting from the treatment of Casper zebrafish
 428 embryos with compounds **2aa** at 96 hpf .a) CTRL- Control; b) embryo treated with 10 μ M c)
 429 embryo treated with 30 μ M; d) embryo treated with 60 μ M; Scale bar 250 μ m. ce: cardiac
 430 edema; yse: yolk sac edema; ct: contracted tail

431

432

433 Conclusions

434

435 We optimized the synthesis of new 2-aryl and 2-alkylimidazolones derivatives, in a one-
 436 pot procedure under microwave irradiations. The latter have shown to be more efficient than
 437 thermal heating by providing similar or slightly better yields of the imidazolones while reducing
 438 the reaction time from 2 hours to 15 minutes. The cytotoxicity of the imidazolones derivatives
 439 was evaluated *in vitro* against tumor cell lines. This family of compounds exhibited interesting
 440 anticancer activity against ER+ and PR+ cell line MCF-7 and HER2+ cell line HepG2. Among
 the synthesized derivatives, compound **2b** expressed the best cytotoxic effect against MCF-7

cell line with an IC₅₀ value of 7.73 μ M, whereas compound **2ab** was the most active against HepG2 cell line with an IC₅₀ of 6.8 μ M. Further studies should be conducted to identify the involvement of protein kinases, overexpressed in these types of tumors, in the pathways responsible for the observed cytotoxicity. Besides their cytotoxic activity on cancer cell lines, these compounds showed biological interactions *in vivo* manifested in morphological abnormalities observed on the development of zebrafish embryos only at high concentrations. Although a limited number of imidazolones derivatives are presented here, it is obvious that a much larger diversity can be achieved through this versatile process to enrich this family of molecules and subsequently conduct a more detailed SAR. This preliminary study showed that this family of compounds may be considered as powerful lead molecules in the further search of novel active and non-toxic anticancer agents.

452

453

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461

Compliance with ethical standards

Conflict of Interest: All authors declare that they have no conflict of interest.

Ethics approval: Adult zebrafish (*Danio rerio*) were used after ethical approval.

Consent to participate: Not applicable

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Supplementary data

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