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Original article

Design, synthesis and evaluation of 3-methylene-substituted indolinones as antimalarials

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ABSTRACT

The design, synthesis and evaluation of 3-methylene-substituted indolinones as falcipain inhibitors and antiplasmodial agents are described. These compounds react readily with thiols via an addition-elimination mechanism, indicating their potential as cysteine protease inhibitors. Several indolinones containing a Leu-*i*-amyl recognition moiety were found to be moderate inhibitors of the *Plasmodium falciparum* cysteine protease falcipain-2, but not of the related protease falcipain-3, and displayed antiplasmodial activity against the chloroquine-resistant *P. falciparum* W2 strain in the low micromolar range. Coupling a 7-chloroquinoline moiety to the 3-methylene-substituted indolinone scaffold led to a significant improvement in antiplasmodial activity.

1. Introduction

Malaria is a serious global health problem, with devastating social and economic consequences in countries where the disease is endemic, due in part to the rapid emergence and spread of parasites that are resistant to well-established antimalarial drugs [1]. As a result, there is an urgent need for novel drugs, preferably acting on underexploited parasite targets in order to delay or overcome the selection of clinical resistance [2]. Cysteine proteases from malaria parasites are of particular interest as therapeutic targets due to their key role in parasite development [3]. *Plasmodium falciparum* expresses four cysteine proteases from the papain family known as falcipains, of which falcipain-2 (FP-2) and falcipain-3 (FP-3) are the most relevant as therapeutic targets [4–6]. As part of our ongoing studies on the design and synthesis of Michael acceptors as cysteine protease inhibitors [4,7–9], we became interested in the study of the 3-methylene-indolinone scaffold (Fig. 1). This scaffold was already used with success for VEGFR, Trk A and CDK inhibitors [10–14]. In particular, indolin-2-ones substituted at C-3 with an aminomethylene group bearing different amino acid moieties were described as antitumor and antibacterial agents [15,16]. However, the potential of 3-methylene-indolinones as antimalarial agents remains to be explored.

2. Results and discussion

2.1. Chemistry

To evaluate the 3-methylene-indolinone scaffold as a Michael acceptor, we first studied the reaction of **1a** with a thiol. Compound **1a** was synthesized from indolin-2-one and DMA-DMF as described in the literature [17].

Interestingly, compound **3** was isolated in 36–50% yields from reaction of **1a** with benzyl mercaptate **2** using different conditions (Scheme 1), consistent with conjugate addition of the thiol or thiolate followed by elimination of the amine moiety (Scheme 1).

A series of 3-(*N*,*N*-dimethylaminomethylene)indolin-2-ones **1b–e** was then synthesized in 77–90% yield. The compounds were obtained as a mixture of *Z*/*E* isomers. The Z/E isomeric ratio (Table 1) was determined from the ^{1}H NMR spectrum on the signal of the olefinic protons, two singlets around 7.4 and 7.6 ppm, respectively.

The next stage was the design of selective falcipain inhibitors, by incorporating the adequate recognition moiety to the double bond. We started by studying the reaction of amines with compound **1a**. Compound **4** (Fig. 2) was obtained in 60% yield by reaction of **1a** with aniline in the presence of hydrochloric acid [16]. However, no reaction occurred when L-phenylalanine and glycine ethyl esters were used. Only when the reaction was performed in the presence of acetic acid at 70 °C, were we able to obtain compounds **5a–b** (Fig. 2).

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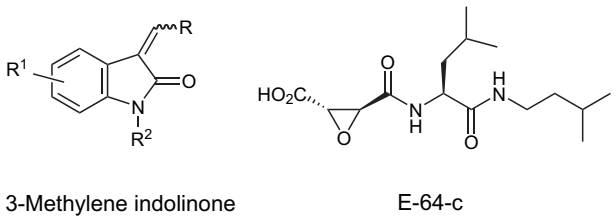


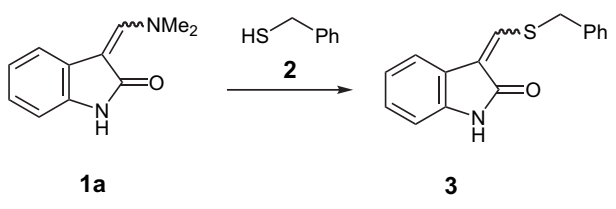
Fig. 1. Chemical structure of 3-methylene-substituted indolinone and E-64c.

A series of compounds **7a**–**i** was designed based on the structure of the epoxide cysteine protease inhibitor E-64c (Fig. 1), which contains a Leu-isoamyl recognition moiety. Phe, homoPhe and Gly residues were also selected in order to modulate the interaction with FP-2. The recognition moiety was obtained starting with different *N*-Boc amino acids (Leu, Phe, homoPhe and Gly) and isoamyl amine in the presence of BOP and THF. Compounds **6a**–**d** were obtained with 62–70% yields after deprotection of the nitrogen with TFA.

Several conditions were used to couple compounds **6a**–**d** to dimethylaminomethylenes **1a**–**e**. However, no reaction occurred using the Moreau method (HCl in MeOH) [16], or AcOH in CH_2Cl_2. Finally, using the method described by Abass [17], compounds **7a**–**i** were obtained in 26–55% yields (Scheme 2) as a mixture of Z/E isomers, which were inseparable by column chromatography. The Z/E isomeric ratio (Table 1) was determined from the ^{1}H NMR spectrum based on the signals of the vinylic protons, which appeared *ca*. 7.43 (d, $J = 12$ Hz) and 7.62 ppm (d, $J = 14$ Hz). The ratio of Z/E isomers varied with the amino acid used in the recognition moiety. Compounds **7a** and **7g** gave a 10:1 Z/E ratio, while compounds **7b**–**e** and **7i** gave a 20:1 mixture of Z/E isomers. In contrast, compounds **7f** and **7h** gave a 3:1 mixture of Z/E isomers. In the case of the homoPhe derivatives, only the Z isomer was obtained probably because of steric hindrance exerted by the homoPhe group in the vicinity of the C=C bond. Overall, the preferred formation of the Z isomer can be ascribed to hydrogen bonding between the NH and the carbonyl oxygen atom of the indole ring (Fig. 3).

The reaction of compound **7a** with benzyl mercaptate **2** was also studied using methanol and triethylamine. In this case, the starting material **7a** was consumed at a slower rate than **1a** suggesting that incorporation of the recognition moiety in the indolin-2-one scaffold reduces the reactivity toward the thiol or thiolate.

In order to further study the potential of 3-methylene-indolin-2-ones as antimalarials we prepared compounds **10a**–**e**, in which the 3-methylene- indolin-2-one scaffold is coupled to a 4-aminoquinoline moiety. The synthesis of derivatives **10a**–**e** involved the preparation of the quinoline intermediate **9** [18] from reaction of 4,7-dichloro quinoline **8**, with 1,3 propane diamine in 94% yield (Scheme 3). Reaction of **9** with 3-(*N*,*N*-dimethylaminomethylene) indolin-2-ones **1a**–**e**, led to **10a**–**e** in 40–70% yield (Scheme 3). In order to study the effect of carbon chain length between



Scheme 1. Reaction of **1a** with benzyl mercaptate **2**. Conditions: AcOH or, CH_2Cl_2/TEA or MeOH/TEA.

Table 1
Yields, Z/E ratio and antiplasmodial activity for compounds **1a**–**e**, **3**, **4**, **5a**–**b**, **7a**–**i** and **10a**–**f**.

Compds	Yield η (%)	Z/E ratio	Falcipain-2 IC_{50}, μM	Falcipain-3 IC_{50}, μM[a]	*P. falciparum* W2 IC_{50}, μM
1a	85	1:1	>50	ND	>50
1b	82	1:1	>50	ND	>50
1c	77	1.25:1	>50	ND	>50
1d	83	1.5:1	>50	ND	>50
1e	90	1.1:1	>50	ND	>50
3	36–50	3:1	>50	ND	>10
4	60	3:1	>50	ND	›10
5a	29	10:1	>50	ND	10.79 ± 1.77
5b	20	10:1	>50	ND	>10
7a	26	10:1	>50	>50	30.73 ± 1.71
7b	40	20:1	44.1 ± 1.3	>50	18.72 ± 1.31
7c	55	20:1	48.5 ± 2.0	>50	15.94 ± 0.46
7d	52	20:1	42.1 ± 2.3	>50	15.66 ± 1.08
7e	55	20:1	>50	>50	23.50 ± 3.49
7f	50	3:1	>50	>50	26.66 ± 3.70
7g	35	10:1	49.9 ± 0.3	>50	18.55 ± 0.40
7h	40	3:1	43.3 ± 0.7	>50	>50
7i	30	20:1	44.0 ± 1.3	>50	13.44 ± 1.77
10a	40	2:1	>50	ND	0.42 ± 0.02
10b	52	2:1	>50	ND	0.20 ± 0.01
10c	62	2:1	>50	ND	0.18 ± 0.02
10d	56	2:1	>50	ND	0.17 ± 0.02
10e	70	10:1	>50	ND	0.29 ± 0.02
10f	68	3:1	ND	ND	0.14 ± 0.01
E-64	–	–	111.7 ± 25.4 (nM)	481.5 ± 6.36 (nM)	1.94 ± 0.004
Chloroquine	–	–	ND	ND	0.14 ± 0.02

[a] ND = not done.

indolinone and quinoline scaffolds, compound **10f** was also synthesized using as starting material 1,2 ethylene diamine (Scheme 3, 68% yield).

2.2. Biological activity

Compounds **1a**–**e**, **3**, **4**, **5a**–**b**, **7a**–**i** and **10a**–**f** were then screened against the parasite cysteine proteases FP-2 and FP-3. Inspection of the data presented in Table 1, shows that the recognition moiety seems to be essential for FP-2 inhibition, as compounds **1a**–**e**, **3**, **4**, **5a**–**b** and **10a**–**f**, lacking the amino acid-isoamyl moiety were devoid of enzyme inhibitory activity. In contrast, none of the compounds tested was active for FP-3. Also worth noting, the presence of a chlorine atom at the indolinone moiety seems to be beneficial for FP-2 inhibition (e.g. **7a** vs **7b**–**d**), but the position of substitution had no major impact on activity (5-Cl, **7b** vs 6-Cl, **7c** vs 7-Cl, **7d**).

Compounds **1a**–**e**, **3**, **4**, **5a**–**b**, **7a**–**i** and **10a**–**f** were then tested against the chloroquine-resistant W2 strain of *P. falciparum* (Table 1). Compounds containing the amino acid-isoamyl moiety,

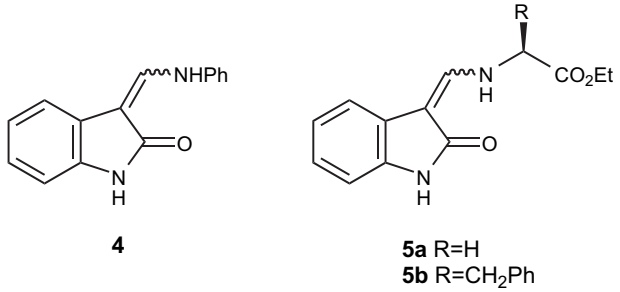
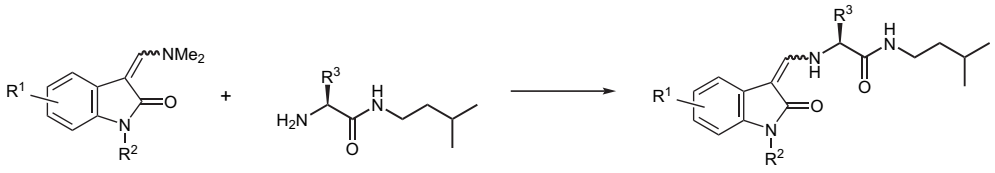


Fig. 2. Chemical structure of compounds **4** and **5a**–**b**.



1a R^1=H; R^2=H
1b R^1=5-Cl; R^2=H
1c R^1=6-Cl; R^2=H
1d R^1=7-Cl; R^2=H
1e R^1=H; R^2=Me

6a R^3=H
6b R^3=$CH_2CH(CH_3)_2$
6c R^3=CH_2Ph
6d R^3=CH_2CH_2Ph

7a R^1=H; R^2=H; R^3=$CH_2CH(CH_3)_2$
7b R^1=5-Cl; R^2=H; R^3=$CH_2CH(CH_3)_2$
7c R^1=6-Cl; R^2=H; R^3=$CH_2CH(CH_3)_2$
7d R^1=7-Cl; R^2=H; R^3=$CH_2CH(CH_3)_2$
7e R^1=H; R^2=Me; R^3=$CH_2CH(CH_3)_2$
7f R^1=H; R^2=H; R^3=H
7g R^1=H; R^2=H; R^3=CH_2CH_2Ph
7h R^1=5-Cl; R^2=H; R^3=CH_2Ph
7i R^1=7-Cl; R^2=H; R^3=CH_2CH_2Ph

Scheme 2. Synthesis of indolinones **7a**–**i**. Conditions: AcOH, 70 °C.

i.e. **7**, had moderate antiplasmodial activity, with IC_{50} values ranging from 13 to 30 μM. In addition, compound **5a**, containing a phenylalanine ethyl ester residue, had an IC_{50} value of 11 μM. These results are similar to those reported for other 2-oxoindole based inhibitors, such as thiosemicarbazone isatin derivatives [19]. Data from Table 1 suggests that FP-2 inhibition might contribute to some extent to the antiplasmodial activity displayed by compounds **7b**–**d** and **7h**–**i**.

Compounds **10a**–**f** did not inhibit FP-2, which can be explained by the absence of a recognition moiety. In contrast, compounds **10a**–**f** displayed potent antiplasmodial activity, with an IC_{50} value of 0.14–0.42 μM against the *P. falciparum* W2 strain (Table 1). Probably, FP-2 inhibition is not the primary mode by which derivatives **10a-f** exert their antiplasmodial activities. It is also worth noting, that variation in carbon chain length between indolinone and quinoline scaffolds had no major impact on activity (3 carbons, **10d** vs 2 carbons, **10f**).

2.3. Docking studies

To gain some insight to the binding mode of our series of inhibitors we studied their molecular interactions with falcipain-2 using GOLD software. Compounds containing Gly, Leu, Phe, and homoPhe (**7a**–**7i**) were docked into the enzyme active site and the results were compared with the crystallized pose of E-64 [5]. The active site of the enzyme is located in a cleft between the structurally distinct domains of the papain like fold, and E-64 interacts with the residues located at S_1, S_2 and S_3 [5].

Each potential inhibitor was energy - minimized and subjected to 2000 docking runs. The top 5 solutions (those presenting the highest fitness score) were visually analyzed. In order to elucidate the inhibitor binding poses we gave special attention to the distances between the potentially reactive carbon and the sulfur atom of the catalytic Cys42 residue, the subsites occupied in the anticipated active site, and hydrophobic interactions between the inhibitors and non polar regions of the falcipain-2 surface.

Our calculations demonstrate a clear difference in the scores obtained for the docking of inhibitors **7a**, **7e** and **7f** (goldscore between 50 and 52) and the most potent compounds of the **7** series (goldscore always superior to 54). When **7a** was docked to falcipain-2, two binding poses were identified. One presents the indolinone moiety in the S'_1 subsite and the Leu-isoamyl in the S_2 pocket, while the other binding pose is inverted, presenting the indolinone moiety well within the S_2 pocket, Leu in S_1 and Leu-isoamyl in the S'_1 subsite. The distances between the reactive carbon and the Cys42 sulfur atom are in the 3.5–5 Å range. A similar inversion in the top binding poses is observed in compound **7e**, with the indolinone moiety occupying S_2 or S_1 subsites and the reactive carbon 3.5 Å distant from the Cys42 sulfur. Another important feature in the active site binding pose of these compounds is that the reactive carbon is never well oriented to the Cys42 sulfur. Interestingly, the less potent compounds (**7e** and **7f**), with the indolinone ring in the S_1 subsite, are the only compounds occupying the top part of this subsite.

With the exception of **7h**, all compounds that demonstrated inhibition, possess the reactive carbon close to the Cys42 sulfur (2.9–3.5 Å) and this carbon is always well oriented to a potential attack. Compounds **7g** and **7i** have exactly the same binding pose,

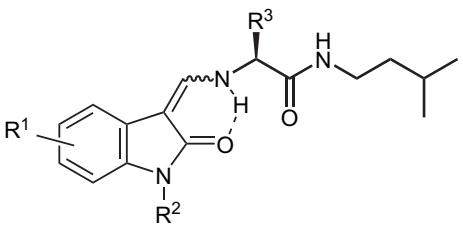
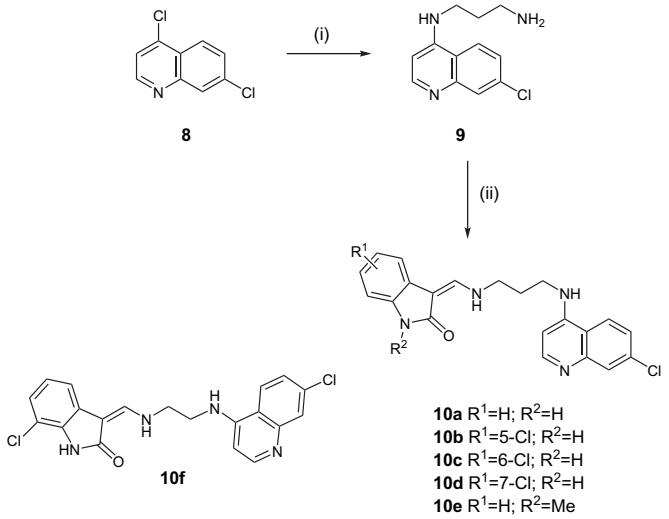


Fig. 3. Hydrogen bond in Z-configuration.



10a R^1=H; R^2=H
10b R^1=5-Cl; R^2=H
10c R^1=6-Cl; R^2=H
10d R^1=7-Cl; R^2=H
10e R^1=H; R^2=Me

Scheme 3. Synthesis of aminoquinoline derivatives **10a**–**f**. Conditions: i) 1,3 propane diamine, 120 °C; ii) **1**, AcOH, 70 °C.

occupying the S_2 and S'_1 subsites, with homoPhe very close to the S_3 subsite. Compound **7h** has a different binding pattern with the distance between the reactive carbon and Cys42 sulfur being around 4.5. This is the only compound that has the indolinone moiety in the S_1 subsite, and the amino acid in P_2 (Phe in this case) is pointing toward the S_3 subsite in a similar way as in the case of E-64.

Our most potent inhibitor, **7d**, is the one that possesses the reactive carbon closer and adequately oriented to the sulfur atom of Cys42 (2.9–3.2 Å), suggesting that this could in principle establish a covalent bond between the enzyme and the inhibitor.

The difference between the inhibition presented by the active studied compounds and the FP-2 inhibitor E-64 can be explained by the different pose that they adopt in the active site. In E-64 the amino acid in P_2 is placed in the S_2 pocket, which does not occur in our compounds.

3. Conclusion

In conclusion, 3-methylene-indolinone-2-ones incorporating a recognition moiety (Leu-*iso*-amyl) for FP-2 inhibition were shown to display moderate inhibitory activity against the parasitic cysteine protease as well as antiplasmodial activity against a chloroquine-resistant strain of *P. falciparum*. Importantly, no FP-2 inhibition could be observed for compounds lacking the recognition moiety, consistent with interaction of the Leu-*iso*-amyl sequence with the S_2 pocket. Furthermore, the antiplasmodial activity could be significantly improved when a 4-aminoquinoline was coupled to the 3-methylene moiety of the indolinone-2-one scaffold, suggesting that these 3-methylene-indolinone-2-ones are potential lead compounds for the development of new antimalarial agents.

4. Experimental protocols

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. High resolution mass spectra (HMRS) were performed in Unidade de Espectrometria de Masas, Santiago de Compostela. Low mass spectra (MS) was performed in Faculdade de Farmácia, Lisbon. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental Analyzer for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield (400 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (J) in hertz.

4.1.1. General procedure for the preparation of 3-(N,N-dimethylaminomethylene) indolin-2-ones **1a–e**

DMF-DMA (2.66 mL, 20.0 mmol) was added to a solution of the appropriate indole (0.78 mmol) in toluene (3 mL). The mixture was stirred for 2–4 h. The precipitate was collected by filtration and washed with dichloromethane.

*4.1.1.1. 3-(N,N-Dimethylaminomethylene) indolin-2-one **1a**.* Obtained in 85% yield, Z/E mixture 1/1. M.p. 210–212 °C. IR (KBr) 3112, 1670, 1580, 1427, 1229, 1114 cm^{-1} ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 10.03 (s br, 1H, NH), 9.95 (s br, 1H, NH), 7.54 (s, 1H), 7.40 (s, 1H), 7.38 (d, 1H, J = 8.0 Hz), 7.26 (d, 1H, J = 8.0 Hz), 6.93 (dd, 1H, J = 8.0 Hz, J = 4.0 Hz), 6.70–6.91 (m, 4H), 6.69 (d, 1H, J = 8.0 Hz), 3.63 (s br, 3H), 3.28 (s, 6H), 3.27 (s br, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 170.80, 165.94, 148.83, 147.93, 137.92, 135.72, 128.74, 122.94, 120.19, 119.78, 119.32, 114.40, 108.59, 107.76, 94.81, 93.02, 43.90. HRMS-ESI-TOF: m/z calc $C_{11}H_{13}N_2O$ (M + 1) 189.1028, found 189.1022.

*4.1.1.2. 3-(N,N-Dimethylaminomethylene) indolin-2-one **1b**.* Obtained in 82% yield, Z/E mixture 2/1. M.p. 204–206 °C. IR (KBr) 3112, 1682, 1593, 1402, 1223, 1153 cm^{-1} ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 10.16 (s br, 1H, NH), 10.07 (s br, 1H, NH), 7.66 (s, 1H), 7.46 (s, 1H), 7.36 (d, 1H, J = 4.0 Hz), 7.30 (d, 1H, J = 4.0 Hz), 6.93 (dd, 1H, J = 8.0 Hz, J = 4.0 Hz), 6.82 (dd, 1H, J = 8.0 Hz, J = 4.0 Hz), 6.74 (d, 1H, J = 8.0 Hz), 6.65 (d, 1H, J = 8.0 Hz), 3.65 (s br, 3H), 3.31 (s, 6H), 3.26 (s br, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 170.62, 165.80, 149.92, 149.08, 136.47, 134.24, 130.97, 124.80, 123.83, 122.09, 120.75, 119.37, 114.14, 109.50, 108.75, 93.83, 92.26, 46.59, 42.30. HRMS-ESI-TOF: m/z calc $C_{11}H_{12}ClN_2O$ (M + 1) 223.0638, found 223.0633.

*4.1.1.3. 3-(N,N-Dimethylaminomethylene) indolin-2-one **1c**.* Obtained in 77% yield, Z/E mixture 1.25/1. M.p. 224–226 °C. IR (KBr) 2907, 1682, 1580, 1421, 1223, 1121 cm^{-1} ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 10.19 (s, 1H, NH), 10.10 (s, 1H, NH), 7.61 (s, 1H), 7.44 (s, 1H), 7.35 (d, 1H, J = 8.0 Hz), 7.26 (d, 1H, J = 8.0 Hz), 6.79–6.83 (m, 2H), 6.76 (d, 1H, J = 2 Hz), 6.69 (d, 1H, J = 2 Hz), 3.63 (s br, 3H), 3.30 (s, 6H), 3.26 (s br, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 170.76, 165.78, 149.41, 148.71, 139.01, 136.66, 127.82, 126.98, 125.62, 122.00, 121.02, 119.25, 118.90, 115.46, 108.32, 107.56, 93.79, 92.13, 46.60, 42.34. HRMS-ESI-TOF: m/z calc $C_{11}H_{12}ClN_2O$ (M + 1) 223.0638, found 223.0633.

*4.1.1.4. 3-(N,N-Dimethylaminomethylene) indolin-2-one **1d**.* Obtained in 83% yield, Z/E mixture 1.5/1. M.p. 230–232 °C. IR (KBr) 3100, 1670, 1587, 1414, 1223, 1127 cm^{-1} ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 10.41 (s, 1H, NH), 10.34 (s, 1H, NH), 7.65 (s, 1H), 7.51 (s, 1H), 7.34 (d, 1H, J = 8.0 Hz), 7.24 (d, 1H, J = 8.0 Hz), 6.96 (d, 1H, J = 8.0 Hz), 6.77–6.88 (m, 3H), 3.66 (s br, 3H), 3.32 (s, 6H), 3.29 (s br, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 170.58, 165.68, 150.21, 149.31, 134.80, 132.62, 130.77, 125.00, 122.29, 121.03, 120.80, 120.46, 118.52, 113.10, 112.92, 112.30, 94.30, 92.72, 46.70, 42.43. HRMS-ESI-TOF: m/z calc $C_{11}H_{12}ClN_2O$ (M + 1) 223.0638, found 223.0633.

*4.1.1.5. 3-(N,N-Dimethylaminomethylene) indolin-2-one **1e**.* Obtained in 90% yield, Z/E mixture 1.1/1. M.p. 58–60 °C. IR (KBr) 3382, 1663, 1574, 1440, 1255, 1159 cm^{-1} ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 7.61 (s, 1H), 7.51 (s, 1H), 7.43 (d, 1H, J = 8 Hz), 7.33 (d, 1H, J = 8 Hz), 7.02 (td, 1H, J = 8.0 Hz, J = 1.2 Hz), 6.94 (td, 1H, J = 8.0 Hz, J = 1.2 Hz), 6.82–6.90 (m, 4H), 3.65 (s br, 3H), 3.31 (s, 6H), 3.28 (s br, 3H), 3.18 (s, 3H), 3.16 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 169.40, 164.09, 149.15, 148.06, 139.15, 136.97, 127.48, 123.00, 122.16, 121.29, 120.32, 120.04, 119.94, 114.25, 107.31, 106.76, 93.88, 92.02, 43.99, 25.73, 25.62. HRMS-ESI-TOF: m/z calc $C_{12}H_{15}N_2O$ (M + 1) 203.1184, found 203.1179.

4.1.2. Procedures for the preparation of indolin-2-one **3**

Method A: The benzyl mercaptane (0.223 mmol) was added to a solution of 3-(*N*,*N*-dimethylaminomethylene) indolin-2-one (0.223 mmol) in acetic acid (3 mL) and the reaction mixture was heated at 70 °C for 12 h. After completion of reaction (monitor by TLC after small workup), the acetic acid was evaporated and the residue was purified by flash chromatography (hexane/ethyl acetate 1:1). The final product was recrystallized in EtOAc and Hexane, yielded 36%.

Method B: The mixture of benzyl mercaptane (0.223 mmol), TEA (0.397 mmol) in CH_2Cl_2 was stirred for 15 min at 0 °C before

addition of 3-(*N*,*N*-dimethylaminomethylene) indolin-2-one (0.265 mmol), then was stired for 48 h at RT. After completion of reaction (monitor by TLC after small workup), water was added and washed with CH_2Cl_2, the organic layer was washed with brine and Na_2SO_4 evaporated, afforded after purification by column chromatography (hexane/ethyl acetate 1:1) 50% yields.

Method C: The mixture of benzyl mercaptane (0.223 mmol), TEA (0.397 mmol) in MeOH was stirred for 15 min at 0 °C before addition of 3-(*N*,*N*-dimethylaminomethylene) indolin-2-one (0.265 mmol), then was stired for 12 h at RT. After completion of reaction (monitor by TLC after small workup), evaporation the methanol, recrystallization from ethyl acetate and hexane was afforded 56% yields.

4.1.2.1. Indolin-2-one ***3***. M.p. 171–173 °C. IR (KBr) 3133, 1682, 1590, 670 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 8.07 (s br, 1H, NH), 7.86 (s, 1H), 7.54 (d, 1H, J = 8.0 Hz), 7.28–7.42 (m, 5H), 7.18 (t, 1H, J = 8.0 Hz), 7.03 (t, 1H, J = 8.0 Hz), 6.85 (d, 1H, J = 8.0 Hz), 4.25 (s. 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.28, 140.35, 139.81, 136.12, 129.12, 129.05, 128.95, 128.93, 128.24, 128.02, 123.88, 122.04, 109.52, 40.07. HRMS-ESI-TOF: m/z calc $C_{16}H_{14}NOS$ (M + 1) 268.0796, found 268.0791.

4.1.3. General procedure for the preparation of indolin-2-ones ***6a–d***

The amino acid (0.432 mmol) and BOP (191 mg, 0.432 mmol) were dissolved in anhydrous THF (4 mL) and stirred with dry TEA (57 μL, 57.4 mmol) for 30 min at room temperature. Then, a mixture of isopentyl amine (37.7 mg, 0.432 mmol) and dry TEA (57 μL, 57.4 mmol) in THF was added under nitrogen atmosphere. The resulting mixture was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (10 mL), washed with water (2 × 15 mL), brine (10 mL), dried over Na_2SO_4, filtered and concentrated. The product was isolated after column chromatography (hexane/ethyl acetate 7:3) in yield 60–70%. Then, the product (0.5 mmol) was dissolved in a 1:1 solution of TFA and DCM (1 mL). The reaction mixture was stirred for 1 h at RT and evaporated under reduced pressure. CCl_4 (3 × 3 mL) was added and evaporated under reduced pressure. This compound was used without further purification.

4.1.4. General procedure for the preparation of indolin-2-ones ***4***, ***5a***, ***5b***, ***7a–i*** *and* ***10a–f***

The appropriate amine (0.39 mmol) was added to a solution of 3-(*N*,*N*-dimethylaminomethylene) indolin-2-one (0.39 mmol) in acetic acid (3 mL) and the reaction mixture was heated at 70 °C for 12 h. After completion of reaction (monitor by TLC after small workup), the acetic acid was evaporated and the residue was purified by flash chromatography (hexane then hexane/ethyl acetate 2:8 to 1:1). The final product was recrystallized in EtOAc and hexane.

4.1.4.1. Indolin-2-one ***4***. Obtained in 60% yield, Z/E mixture 5/1. M.p. 160–162 °C. IR (KBr) 3230, 3113, 1672, 1572 cm^{-1} ^1H NMR (400 MHz, MeOD) δ 8.34 (s, 1H, NH), 7.79 (s, 1H, NH), 7.49 (d, 1H, J = 8.0 Hz), 7.38 (m, 2H), 7.26 (d, 2H, J = 8.0 Hz), 6.91–7.10 (m, 4H). ^{13}C NMR (100 MHz, MeOD) δ 192.52, 141.51, 139.43, 138.23, 131.20, 125.67, 125.02, 122.58, 121.71, 117.94, 117.24, 110.99, 24.69. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{15}H_{13}N_2O$ 237.1028, found 237.1148.

4.1.4.2. Indolin-2-one ***5a***. Obtained in 29% yield, Z/E mixture 10/1. M.p. 162–164 °C. IR (KBr) 3420, 3250, 1710, 1680 cm^{-1}. Z-isomer - ^1H NMR (400 MHz, $CDCl_3$) δ 9.15 (s br, 1H, NH), 9.00 (s br, 1H, NH), 7.60 (d, 1H, J = 12.0 Hz), 7.55 (s, 1H), 7.48 (d, 1H, J = 8.0 Hz), 7.33 (t, 1H, J = 8.0 Hz), 7.26 (t, 1H, J = 8.0 Hz), 4.54 (q, 2H, J = 8.0 Hz), 4.40 (d, 2H, J = 4.0 Hz), 1.59 (t, 3H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.13, 146.84, 135.39, 124.56, 123.58, 121.16, 115.59, 109.77, 61.91, 49.80, 14.16. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{13}H_{15}N_2O_3$ 247.1083, found 247.1077.

4.1.4.3. Indolin-2-one ***5b***. Obtained in 20% yield, Z/E mixture 10/1. M.p. 146–148 °C. IR (KBr) 3205, 3110, 1739, 1678 cm^{-1}. Z-isomer - ^1H NMR (400 MHz, $CDCl_3$) δ 9.01 (s br, 1H, J = 8 Hz NH), 8.79 (s, 1H, NH), 7.24–7.34 (m, 6H), 7.09–7.17(m, 3H), 7.04 (t, 1H, J = 8.0 Hz), 6.94 (t, 2H, J = 8.0 Hz), 4.25 (q, 2H, J = 8.0 Hz), 3.32 (dd, 1H, J = 12.0 Hz, J = 4.0 Hz), 3.13 (dd, 1H, J = 12.0 Hz, J = 8.0 Hz), 1.29 (t, 3H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.70, 170.54, 144.90, 135.69, 134.63, 129.63, 128.93, 127.51, 124.73, 123.62, 120.98, 115.54, 109.66, 97.48, 63.07, 62.09, 40.23, 14.24. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{20}H_{21}N_2O_3$ 337.1552, found 337.1547.

4.1.4.4. Indolin-2-one ***7a***. Obtained in 26% yield; Z/E mixture 10/1. M.p. 174–176 °C. IR (KBr) 3450, 3024, 2928, 1675, 1606, 1211 cm^{-1}. Z-isomer - ^1H NMR (400 MHz, $CDCl_3$): δ 8.72–8.77 (m, 1H), 8.39 (s, 1H), 7.43 (d, 1H, J = 12.0 Hz), 7.25 (d, 1H, J = 4.0 Hz), 7.06 (t, 1H, J = 8.0 Hz), 6.98 (t, 1H, J = 8.0 Hz), 6.92 (d, 1H, J = 8.0 Hz), 6.21 (s br, 1H), 3.89–3.94 (m, 1H), 3.27 (q, 2H, J = 8.0 Hz), 1.86–1.89 (m, 1H), 1.71–1.74 (m, 2H), 1.55–1.59 (m, 1H), 1.37 (q, 2H, J = 8.0 Hz), 0.97 (dd, 6H, J = 8.0 Hz, J = 4.0 Hz), 0.88 (dd, 6H, J = 8.0 Hz, J = 4.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.74, 145.29, 135.65, 124.36, 124.16, 121.39, 115.95, 109.71, 98.36, 61.68, 41.77, 38.42, 38.27, 26.05, 24.84, 23.24, 22.54, 22.53, 21.46. Anal. Cald for $C_{20}H_{29}N_3O_2$: C, 69.94; H, 8.51; N, 12.23; O 9.32. Found: C, 69.35; H, 8.60; N, 11.76.

4.1.4.5. Indolin-2-one ***7b***. Obtained in 40% yield; Z/E mixture 20/1. M.p. 173–175 °C. IR (KBr) 3316, 3172, 2956, 1657 cm^{-1}; Z-isomer - ^1H NMR (400 MHz, $CDCl_3$): δ 8.82–8.88 (m, 1H), 8.12 (s, 1H), 7.45 (d, 1H, J = 12.0 Hz), 7.24 (s, 1H), 7.02 (dd, 1H, J = 8.0 Hz, J = 4.0 Hz), 6.83 (d, 1H, J = 8.0 Hz), 6.06 (s br, 1H), 3.89–3.93 (m, 1H), 3.28 (q, 2H, J = 8.0 Hz), 1.86–1.90 (m, 1H), 1.70–1.80 (m, 2H), 1.56–1.60 (m, 1H), 1.38 (q, J = 8.0 Hz, 2H), 0.97 (dd, J = 8.0 Hz, J = 4.0 Hz, 6H), 0.89 (d, J = 4.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 171.49, 170.84, 146.08, 134.17, 126.39, 126.11, 123.25, 115.72, 110.52, 97.28, 61.33, 42.00, 38.31, 25.99, 24.71, 23.08, 22.46, 21.62. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{20}H_{29}ClN_3O_2$ 378.1948, found 378.1943.

4.1.4.6. Indolin-2-one ***7c***. Obtained in 55% yield; Z/E mixture 20/1. M.p. 193–195 °C. IR (KBr) 3436, 3016, 2932, 1735, 1621 cm^{-1}; Z-isomer - ^1H NMR (400 MHz, $CDCl_3$): δ 8.74–8.79 (m, 1H, NH), 8.15 (s, 1H), 7.42 (d, 1H, J = 12.0 Hz), 7.14 (d, 1H, J = 8.0 Hz), 6.96 (dd, 1H, J = 8.0 Hz, J = 1.5 Hz), 6.92 (s, 1H), 6.06 (s br, 1H), 3.88–3.93 (m, 1H), 3.25–3.31 (m, 2H), 1.80–1.90 (m, 1H), 1.69–1.74 (m, 3H), 1.56–1.57 (m, 1H), 1.38 (q, 2H, J = 8.0 Hz), 0.97 (dd, 6H, J = 8.0 Hz, J = 4.0 Hz), 0.89 (d, 6H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 171.43, 170.84, 145.58, 136.24, 129.41, 122.94, 121.40, 116.70, 110.08, 97.63, 61.68, 41.82, 38.45, 38.32, 26.08, 24.85, 23.23, 22.56, 21.52. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{20}H_{29}ClN_3O_2$ 378.1948, found 378.1943.

4.1.4.7. Indolin-2-one ***7d***. Obtained in 52% yield; Z/E mixture 20/1. M.p. 168–170 °C. IR (KBr) 3285, 2928, 1681, 1613 cm^{-1}; Z-isomer - ^1H NMR (400 MHz, $CDCl_3$): δ 8.87–8.92 (m, 1H, NH), 8.41 (d, 1H, J = 8.0 Hz), 7.48 (d, 1H, J = 12.0 Hz), 7.14 (d, 1H, J = 8.0 Hz), 7.04 (d, 1H, J = 8.0 Hz), 6.92 (t, 1H, J = 8.0 Hz), 6.19 (s br, 0.5H, NH), 5.98–6.00 (m, 0.5H, NH), 3.91–3.93 (m, 1H), 3.74–3.76 (m, 2H), 1.61–1.90 (m, 3H), 1.55–1.60 (m, 1H), 1.32–1.41 (m, 2H), 0.96 (dd, 6H, J = 8.0 Hz, J = 4.0 Hz), 0.88 (d, 6H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 171.39, 170.47, 146.41, 132.93, 125.86, 123.58, 122.14, 114.98, 114.13, 98.22, 68.11, 61.66, 41.81, 38.42, 38.31, 26.06, 24.83, 23.21, 22.54, 21.51. HRMS-ESI-TOF (M^+ + 1) calcd for $C_{20}H_{29}ClN_3O_2$ 378.1948, found 378.1943.

4.1.4.8. Indolin-2-one ***7e***. Obtained in 55% yield; Z/E mixture 20/1. M.p. 183–185 °C. IR (KBr) 3292, 3022, 2971, 1673, 1622 cm^{-1}; Z-isomer - ^1H NMR (400 MHz, $CDCl_3$): δ 8.72–8.78 (m, 1H), 7.43 (d, 1H, J = 12.0 Hz), 7.27 (d, 1H, J = 4.0 Hz), 7.11 (td, 1H, J = 8.0 Hz, J = 4.0 Hz), 7.04 (t, J = 8.0 Hz, 1H), 6.84 (d, 1H, J = 8.0 Hz), 6.44 (s br, 1H), 3.91–3.96 (m 1H), 3.27 (s, 3H), 3.26–3.32 (m, 2H), 1.88–1.91 (m, 1H), 1.71–1.74 (m, 2H), 1.58–1.60 (m, 1H), 1.39 (q, 2H, J = 8.0 Hz), 0.95 (dd, 6H, J = 8.0 Hz, J = 4.0 Hz), 0.87 (dd, 6H, J = 8.0 Hz, J = 2.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 171.89, 169.13, 144.62, 138.23, 124.07, 123.37, 121.31, 115.62, 107.91, 98.26, 61.60, 41.75, 38.42, 38.21, 26.01, 25.59, 24.83, 23.27, 22.53, 22.51, 21.36. HRMS-ESI-TOF ($M^+ + 1$) calcd for $C_{21}H_{32}N_3O_2$ 358.2495, found 358.2481.

4.1.4.9. Indolin-2-one ***7f***. Obtained in 50% yield; Z/E mixture 3/1. M.p. 203–205 °C. IR (KBr) 3318, 3022, 2958, 1667, 1603 cm^{-1}; Z-isomer - ^1H NMR (400 MHz, DMSO-d_6): δ 10.18 (s, 1H, NH), 8.76–8.83 (m, 1H), 8.01–8.04 (m, 1H), 7.80 (d, 1H, J = 13.12 Hz), 7.26 (d, 1H, J = 8.0 Hz), 6.76–6.97 (m, 3H), 3.99 (d, 2H, J = 8.0 Hz), 3.11 (q, 2H, J = 8.0 Hz), 1.55–1.62 (m, 1H), 1.32 (q, 2H, J = 8.0 Hz), 0.87 (d, 6H, J = 8.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.59, 168.14, 147.98, 135.91, 125.28, 122.32, 119.77, 115.10, 108.69, 95.40, 50.22, 38.08, 36.90, 25.13, 22.40. Anal. Cald for $C_{16}H_{21}N_3O_2$: C, 66.88; H, 7.37; N, 14.62. Found: C, 66.49; H, 7.25; N, 14.18.

4.1.4.10. Indolin-2-one ***7g***. Obtained in 35% yield; Z/E mixture 10/1. M.p. 134–136 °C. IR (KBr) 3312, 2941, 1661, 1593 cm^{-1}; Z-isomer - ^{1}HNMR (400 MHz, $CDCl_3$): δ 8.81–8.86 (m, 1H, NH), 8.14 (s br, 1H, NH), 7.36 (d, 1H, J = 12.0 Hz), 7.19–7.31 (m, 6H), 7.08 (t, 1H, J = 8.0 Hz), 7.01 (t, 1H, J = 8.0 Hz), 6.94 (d, 1H, J = 8.0 Hz), 6.13 (s br, 1H), 3.75–3.80 (m, 1H), 3.26 (q, 2H, J = 8.0 Hz), 2.82–2.85 (m, 1H), 2.70–2.73 (m, 1H), 2.46–2.48 (m, 1H), 2.09–2.13 (m, 1H), 1.50–1.56 (m, 1H), 1.37 (q, 2H, J = 8.0 Hz), 0.88 (d, 6H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 170.88, 170.59, 145.25, 139.86, 135.52, 128.65, 128.56, 126.40, 124.13, 121.33, 115.87, 109.57, 98.43, 62.13, 38.31, 38.17, 34.07, 31.71, 25.93, 22.42. Anal. Cald for $C_{24}H_{29}N_3O_2$: C, 73.63; H, 7.47; N, 10.73. Found: C, 73.84; H, 7.53; N, 10.45.

4.1.4.11. Indolin-2-one ***7h***. Obtained in 40% yield; Z/E mixture 3/1; M.p. 242–244 °C. IR (KBr) 3436, 3024, 1729, 1681 cm^{-1}; Z-isomer – ^1H NMR (400 MHz, DMSO-d_6): δ 10.32 (s, 1H), 9.00–9.05 (m, 1H, NH), 8.22 (t, 1H, J = 8.0 Hz,), 7.82 (d, 1H, J = 12.0 Hz), 7.18–7.31 (m, 6H), 6.89 (dd, 1H, J = 8.0 Hz, J = 4.0 Hz), 6.74 (d, 1H, J = 8.0 Hz), 4.22–4.27 (m, 1H), 2.99–3.15 (m, 4H), 1.44–1.49 (m, 1H), 1.25 (q, 2H, J = 8.0 Hz), 0.84 (d, 6H, J = 4.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): 169.44, 169.37, 147.16, 136.70, 134.48, 129.46, 128.30, 127.05, 126.63, 124.21, 121.67, 115.04, 109.82, 94.84, 62.01, 37.86, 36.82, 24.94, 22.36, 22.34. Anal. Cald for $C_{23}H_{26}ClN_3O_2$: C, 67.06; H, 6.36; N, 10.20. Found: C, 66.87; H, 5.96; N, 9.81.

4.1.4.12. Indolin-2-one ***7i***. Obtained in 30% yield; Z/E mixture 20/1. M.p. 66–68 °C. IR (KBr) 3304, 3019, 1681, 1609 cm^{-1}; Z-isomer ^1H NMR (400 MHz, $CDCl_3$): δ 9.29 (s br, 1H, NH), 9.12–9.18 (m, 1H, NH), 7.48 (d, 1H, J = 12.0 Hz), 7.26–7.30 (m, 3H), 7.17–7.21 (m, 3H), 7.13 (d, 1H, J = 8.0 Hz,), 7.04 (d, 1H, J = 8.0 Hz), 6.92 (t, 1H, J = 8.0 Hz), 6.46–6.48 (m, 1H), 3.86–3.92 (m, 1H), 3.25–3.30 (m, 2H), 2.65–2.85 (m, 2H), 2.40–2.50 (m, 1H), 2.12–2.16 (m, 1H), 1.55–1.58 (m, 1H), 1.39 (q, J = 8.0 Hz, 2H), 0.88 (d, 6H, J = 4.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 170.80, 170.29, 146.64, 139.91, 133.06, 128.70, 128.54, 126.45, 125.95, 123.45, 122.04, 115.03, 114.00, 98.07, 62.09, 38.32, 38.30, 34.35, 31.70, 25.98, 22.46. HRMS-ESI-TOF ($M^+ + 1$) calcd for $C_{24}H_{29}ClN_3O_2$ 426.1948, found 426.1943.

4.1.4.13. Indolin-2-one ***10a***. Obtained in 40% yield; Z/E mixture 2/1. mp: 133–135 °C. IR (KBr): 3275, 3207, 1652, 1592, 1449 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 10.18 (s, 1H, NH), 8.88 (t br, 1H, J = 8 Hz, NH), 8.39 (d, 1H, J = 4 Hz), 8.27 (d, 1H, J = 8 Hz), 7.90 (d, 1H, J = 12 Hz), 7.79 (s, 1H), 7.45–7.47 (m, 1H), 7.38 (s br, 1H, NH), 7.25 (d, 1H, J = 8 Hz), 6.75–6.90 (m, 3H), 6.49 (d, 1H, J = 4 Hz), 3.48 (m, 2H), 3.33 (m, 2H), 1.98 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 169.82, 151.73, 150.01, 148.79, 147.80, 135.56, 133.49, 127.11, 125.31, 124.09, 124.04, 122.04, 119.67, 117.90, 114.98, 108.71, 98.73, 94.72, 46.08, 39.08, 29.43. HRMS-EI: calcd for $C_{21}H_{19}ClN_4O$ 378.1247, found 378.1248.

4.1.4.14. Indolin-2-one ***10b***. Obtained in 52% yield; Z/E mixture 2/1. M.p. 278–280 °C. IR (KBr) 3287, 1668, 1560, 1456 cm^{-1}; ^1H NMR (400 MHz, MeOD): δ 8.31 (d, 1H, J = 4 Hz, 1H), 8.11 (d, 1H, J = 12 Hz), 7.77 (s, 1H), 7.70 (d, 1H, J = 2 Hz), 7.39 (dd, 1H, J = 12, 4 Hz), 7.11 (d, 1H, J = 2 Hz), 6.89 (dd, 1H, J = 12, 4 Hz), 6.78 (s, 1H), 6.67 (d, 1H, J = 8 Hz), 3.61 (m, 4H), 2.13 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 171.74, 154.67, 150.13, 148.37, 138.29, 135.12, 128.21, 127.16, 127.13, 124.86, 124.11, 123.19, 119.36, 117.93, 116.03, 111.13, 99.67, 95.97, 48.22, 41.88, 30.14. HRMS-EI: calcd for $C_{21}H_{19}Cl_2N_4O$ 412.0858, found 412.0848.

4.1.4.15. Indolin-2-one ***10c***. Obtained in 62% yield; Z/E mixture 2/1; mp: 265–267 °C. IR (KBr): Cm^{-1} 3292, 3216, 1679, 1586, 1450, 1220; ^1H NMR (400 MHz, DMSO-d_6): δ 10.33 (s, 1H, NH), 8.95 (t br, 1H, J = 8 Hz, NH), 8.39 (d, 1H, J = 4 Hz), 8.26 (d, 1H, J = 8 Hz), 7.97 (d, 1H, J = 12 Hz), 7.79 (s, 1H), 7.44 (d, 1H, J = 12 Hz), 7.40 (s br, 1H, NH), 7.24 (d, 1H, J = 8 Hz), 6.87 (d, 1H, J = 8 Hz), 6.76 (d, 1H, J = 8 Hz), 6.49 (d, 1H, J = 4 Hz), 3.48 (m, 2H), 3.33 (m, 2H), 1.98 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.58, 151.82, 150.18, 149.00, 148.70, 136.67, 133.51, 127.54, 126.01, 124.37, 124.15, 119.43, 119.22, 117.51, 116.14, 108.57, 98.82, 93.79, 46.34, 39.52, 29.37. HRMS-ESI-TOF ($M^+ + 1$) calcd for $C_{21}H_{19}Cl_2N_4O$ 413.0936, found 413.0930.

4.1.4.16. Indolin-2-one ***10d***. Obtained in 56% yield; Z/E mixture 2/1. M.p. 123–125 °C. IR (KBr) 3275, 1672, 1568, 1461 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.58 (s, 1H, NH), 9.04 (t br, 1H, J = 8 Hz, NH), 8.39 (d, 1H, J = 4 Hz), 8.26 (d, 1H, J = 8 Hz), 8.02 (d, 1H, 12 Hz), 7.79 (s, 1H), 7.47 (d, 1H, J = 8 Hz), 7.39 (s br, 1H, NH), 7.22 (d, 1H, J = 8 Hz), 6.91 (d, 1H, J = 8 Hz), 6.84 (d, 1H, J = 8 Hz), 6.49 (d, 1H, J = 4 Hz), 3.51 (m, 2H), 3.35 (m, 2H), 1.99 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.89, 151.42, 149.55, 148.75, 148.64, 133.00, 132.20, 126.94, 126.91, 123.62, 123.57, 121.01, 120.46, 117.04, 112.97, 112.61, 98.17, 93.85, 45.96, 39.40, 28.76. MS-ESI m/z: $C_{21}H_{19}Cl_2N_4O$ 412.96 (M^+).

4.1.4.17. Indolin-2-one ***10e***. Obtained in 70% yield; Z/E mixture 10/1. M.p. 63–65 °C. IR (KBr) 3275, 1681, 1575, 1445 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.80 (t br, 1H, J = 8 Hz, NH), 8.19 (s, 1H, NH), 8.02 (d, 1H, J = 8 Hz), 7.94 (d, 1H, J = 8 Hz), 7.70 (s, 1H), 7.47 (d, 1H, J = 12 Hz), 7.18 (d, 1H, J = 8 Hz), 7.16 (d, 1H, J = 8 Hz), 7.08 (t, 1H, J = 8 Hz), 6.97 (t, 1H, J = 8 Hz), 6.84 (d, 1H, J = 8 Hz), 6.07 (d, 1H, J = 4 Hz), 3.52 (m, 2H), 3.40 (m br, 2H), 3.29 (s, 3H), 2.05 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.98, 153.14, 146.47, 145.55, 142.30, 137.66, 137.55, 126.32, 123.93, 123.84, 123.30, 122.69, 121.18, 115.99, 115.13, 107.80, 97.63, 96.01, 46.78, 40.63, 29.39, 25.66. HRMS-EI: calcd for $C_{22}H_{21}ClN_4O$ 392.1404, found 392.1403.

4.1.4.18. Indolin-2-one ***10f***. Obtained in 68% yield; Z/E mixture 3/1. M.p. >300 °C. IR (KBr) 3269, 1682, 1559, 1430 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 10.55 (s, 1H, NH), 9.09 (t, 1H, J = 8 Hz, NH), 8.41 (d, 1H, J = 8 Hz), 8.25 (d, 1H, J = 8 Hz), 7.94 (d, 1H, J = 12 Hz), 7.79 (s, 1H), 7.47 (s br, 1H, NH), 7.45 (d, 1H, J = 8 Hz), 7.13 (d, 1H, J = 4 Hz), 6.89 (d, 1H, J = 8 Hz), 6.82 (t, 1H, J = 8 Hz), 6.65 (d, 1H, J = 4 Hz), 3.65 (m br, 2H), 3.52 (m br, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.18, 151.99, 150.01, 149.08, 133.46, 132.67, 127.64, 127.42, 124.25, 124.03, 121.51, 120.91, 117.50, 113.38, 113.08, 112.70, 99.16, 94.63, 46.91, 43.51. HRMS-EI: calcd for $C_{20}H_{16}Cl_2N_4O$ 398.0701, found 398.0704.

4.2. Pharmacology

4.2.1. Falcipain-2 and -3 assays

Both enzymes were assayed at 25 °C using Z-Leu-Arg-AMC as substrate at a concentration of 25 μM in 100 mM sodium acetate pH 6.0, 5 mM DTT, 0.75% DMSO. One microliter of the compound dissolved in DMSO was diluted into 100 μL of assay buffer. Fifty microliters of the enzyme in assay buffer were added and the resulting mixture was incubated for 10 min. The reaction was initiated by addition of 50 μL of assay buffer containing the substrate and read immediately in a Fluoroskan Ascent microplate spectrofluorometer. Every compound was assayed at eight different concentrations, with 50 μM being the highest inhibitor concentration; all other concentrations were created by exponential five-fold dilution. The IC_{50} values were determined by plotting the percentage of inhibition relative to the control reaction in the absence of inhibitor over the logarithmic compound concentration by using the equation $v/v_0 = 1/(1 + 10^{\log[I]}/IC_{50})$. Data analysis was done with the program GraphPad Prism 4 (GraphPad Software).

4.2.2. In vitro antiplasmodial activity in human red blood cells

Human red blood cells infected with 1% ring stage *P. falciparum* strains synchronized with 5% sorbitol were incubated with tested compounds (stock solutions in DMSO) in 96 well plates at 37 °C for 48 h in RPMI-1640 medium, supplemented with 25 mM HEPES pH 7.4, 10% heat inactivated human serum (or 0.5% Albumax, 2% human serum), and 100 uM Hypoxanthine under an atmosphere of 3% O_2, 5% CO_2, 91% N_2. After 48 h the cells were fixed in 2% HCHO in PBS, transferred into PBS with 100 mM NH_4Cl, 0.1% Triton X-100, 1 nM YOYO-1, and then analyzed in a flow cytometer (FACSort, Beckton Dickinson; EX 488 nm, EM 520 nm) IC_{50} were calculated using GraphPad PRISM software.

4.3. Computational studies

Molecular docking studies were performed, using the GOLD software [20] (version 5) to predict the interactions and binding modes of **7** series of synthesized inhibitors in the falcipain-2 active site, and to evaluate their relative binding affinities. The 3D structure coordinates of falcipain-2 were obtained after a search in the Protein Data Bank and electing the structure with code 3BPF with a resolution of 2.9 Å [5]. To prepare the enzyme for the docking studies, the E-64 inhibitor included in the 3BPF structure was removed, hydrogen atoms were added to falcipain-2, crystallographic waters were removed and the protonation states were correctly assigned using the Protonate-3D tool within the Molecular Operating Environment (MOE) 2009.10 software package [21]. To evaluate and validate the effectiveness of our model system and the performance of the docking protocol, a set of studies was first performed using the co-crystallized inhibitor to reproduce the experimental binding pose. The best docking pose obtained was overlaid with the experimental crystal structure showing a minimal deviation of its atomic coordinates (RMSD < 2 Å). Since the validation study revealed that our model and software protocol could accurately reproduce the experimentally binding mode of the E-64 inhibitor, a series of synthesized 3-methylene-substituted indolinones were covalently docked into the falcipain-2 binding site using the Goldscore fitness function [22].

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