Research paper

Synthesis of new derivatives of boehmeriasin A and their biological evaluation in liver cancer

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Two series of boehmeriasin A analogs have been synthesized in short and high yielding processes providing derivatives differing either in the alkaloid's pentacyclic scaffold or its peripheral substitution pattern. These series have enabled, for the first time, comparative studies into key biological properties revealing a new lead compound with exceptionally high activity against liver cancer cell lines in the picomolar range for both well (Huh7, Hep3B and HepG2) and poorly (Mahlavu, FOCUS and SNU475) differentiated cells. The cell death was characterized as apoptosis by cytochrome-C release, PARP protein cleavage and SubG1 cell cycle arrest. Subsequent testing associated apoptosis via oxidative stress with in situ formation of reactive oxygen species (ROS) and altered phospho-protein levels. Compound 19 decreased Akt protein phosphorylation which is crucially involved in liver cancer tumorigenesis. Given its simple synthetic accessibility and intriguing biological properties this new lead compound could address unmet challenges within liver cancer therapy.

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1. Introduction

Primary liver cancer (hepatocellular carcinoma, HCC), is the sixth most frequent cancer type and the second highest cause of cancer-related death worldwide [1]. Viral infections with hepatitis B or C, alcoholic injury, obesity and other factors inducing cirrhosis and chronic liver disease are additional major risk contributors for HCC [2–4]. It is also expected that the rate of liver cancer and associated deaths will increase in the coming years due to the global epidemic of non-alcoholic fatty liver disease (NAFLD) [5]. Despite these well-known etiological factors, liver cancer remains one of the most lethal types of cancer, due to the very limited therapeutic options that offer significant clinical benefits [6]. Liver resection, transplantation and chemoembolization are still the most relied upon options, however, not all the patients can meet the criteria for these treatments [7]. Meanwhile, patients with advanced HCC suffer from the lack of effective therapy. The first FDA approved drug Sorafenib (5, Fig. 1), which is a multi-kinase inhibitor, can only improve patients’ median survival for about 3 months [8]. Very recently, the closely related multi-kinase inhibitor Regorafenib was used in patients as a second line treatment. The mean survival period was 10 months compared to a placebo (~8 months) [9].

To address these challenges in the arena of liver cancer [10], we decided to investigate the chemical synthesis and biological evaluation of new analogs of the known plant alkaloid boehmeriasin A. Boehmeriasin A (1, Fig. 1) is a pentacyclic phenanthroquinolizidine alkaloid recently isolated from the ethanolic extract of boehmeria siamensis Craib [11], a plant that has long been used in south-east Asia for the treatment of rheumatism as well as skin diseases such as urticaria (hives). Through extensive biological studies, it has been revealed that 1 possesses significant activity against several cancer cell lines commonly exceeding the potency of Taxol by at least tenfold. The nanomolar cytotoxic activity of boehmeriasin A was established for various cancer cells, originating from lung, colon, breast, prostate, kidney cancers, and leukemia [12]. In addition to this anti-cancer activity boehmeriasin A also causes G1 cell cycle arrest, cellular differentiation, affects cellular
morphology and results in lipid droplet accumulation in breast cancer cells [13]. It was furthermore found that boehmeriasin A shares this promising anti-cancer activity with other members of the phenanthroquinolizidine and -pyrrolizidine alkaloids such as tylophorine (2), antofine (3) and cryptopleurine (4) (Fig. 1) [14–21]. Topoisomerases and SIRT2 were proposed as potential biological targets of Boehmeriasin A [24].

Driven by the promising bioactivity reports, many successful total syntheses of these intriguing cytotoxic alkaloids have been disclosed [12,22–24]. Despite these efforts neither the molecular target nor the mode of action of these important structures have been identified and detailed SAR studies are lacking, which severely hamper development of further boehmeriasin A based leads. To establish this missing key data, we wished to evaluate boehmeriasin A as well as several new analogs in the context of human epithelial cancers especially in liver cancer.

1.1. Analog synthesis

Upon analysis of the parent pentacyclic scaffold of boehmeriasin A (1) and its closely related natural products (2–4) we decided to design and synthesize two series of target compounds to study their structure-activity relationship. The first series would retain the oxygenation pattern of boehmeriasin A but introduce alterations in the connectivity of the fused ring system, whereas the second series would conserve the original pentacyclic framework while modifications to the embedded quinolizidine system would be made.

To create these two series, we decided to exploit a synthetic route to boehmeriasin A itself which we had previously developed (Scheme 1) [24]. The route is based on an efficient 7-step sequence in which a Perkin reaction is followed by an esterification to yield building block 8 that was then converted into the phenanthrene unit 9 by a FeCl3-mediated oxidation. The pendant ester functionality was then reduced to an alcohol and activated as the corresponding chloride (10) to allow coupling with pipercholic acid (11). Treatment of 12 with polyphosphoric acid (PPA) leads to ring closure via a Friedel-Crafts acylation reaction and furnishes ketone 13 that upon full reduction gives the desired natural product 1. This approach was deemed very attractive as it had been shown to be robust and scalable and furthermore allows the introduction of the desirable variations on different moieties of the parent structure of 1.

The synthesis of the first analog series utilized intermediates 8 and 9 that were independently elaborated by amide coupling with aminopropyl imidazole (14, Scheme 2). The aminopropyl imidazole was selected as a hydrophobic appendage to mimic the quinolizidine substructure of 1 and to improve bioavailability. Furthermore, it was intended to modulate the planarity of the phenanthrene system by omitting the central C-C bond in some of the analogs, which would enable us to evaluate the likelihood of these entities acting via DNA intercalation.

Compounds 8 and 9 were hydrolyzed under basic conditions rendering the corresponding carboxylic acid derivatives that were subsequently activated by CDI and coupled with aminopropyl imidazole (14) to furnish the desired amides 15 and 16 in good yield. In addition, both amides were treated with LAH (2.5 equiv.) to obtain the corresponding amines 17 and 18 allowing to establish the effect of having an amide versus an amine incorporated into these structures, which could have implications due to altered H-bonding patterns.

With the first analog series in hand we turned our attention to a second series in which we envisaged studying alterations on the

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**Fig. 1.** Structures of racemic boehmeriasin A 1, related alkaloids (2–4) and sorafenib.

**Scheme 1.** Previous synthesis of racemic boehmeriasin A (1).
pentacyclic framework of boehmeriasin A. Specifically, we decided to explore manipulations of the carbonyl of 13 by means of its reduction to the corresponding alcohol derivatives. The synthesis of the desired alcohols was readily accomplished by reduction of 13 by LAH (2.0 equiv.) giving a diastereomeric mixture of alcohol products 19a and 19b in a 1:2 ratio. Pleasingly, it was possible to separate this mixture by preparative TLC to undertake characterization and subsequent biological evaluation of the individual diastereomers. Additionally, single crystal X-ray diffraction experiments were used to identify the major diastereomer 19b as the trans-alcohol product (Scheme 3).

Together with boehmeriasin A (1) and its carbonyl derivative 13, these individual alcohol derivatives 19a and 19b therefore represent the second series of analogs available for detailed biological testing (Fig. 2).

2. Biological studies

2.1. Biological activities of first series of boehmeriasin A analogs

Commencing our studies with boehmeriasin A analogs 15–18 we analyzed their cellular toxicity against different hepatocellular carcinoma cells, namely Huh7 and Mahlavu. As pointed out earlier we wished to establish whether simplified structures possessing a conformationally open ring system would still impart significant cytotoxicity, whilst improving solubility by introduction of an imidazole appendage. However, to our surprise it was found that these analogs displayed either very moderate activity (compounds 16 and 17 IC50 10–54 μM) or no activity (compounds 15 and 18, Table 1). Additionally, the presence of an amide or amine linkage as well as the central biaryl bond appears to be irrelevant with regards to activity against these cell lines. Whilst somewhat unexpected this finding establishes clearly that the pentacyclic framework of the boehmeriasins is crucial for their sub-micromolar biological activity which had not been demonstrated before. This furthermore highlights the importance of the embedded quinolizidine substructure and prompted us to evaluate analogs of our second series next.

2.2. Cytotoxicity of second series of boehmeriasin A derivatives

To verify the anticipated biological activity of the parent boehmeriasin A (1) and its hydroxy analog 19 (as 1:2 mixture of diastereomers) an initial screen against different epithelial cancer cells, namely liver (Huh7), breast (MCF7) and colon (HCT116) carcinoma cells was performed. The cytotoxicity of each compound was assessed by sulforhodamine B (SRB) assay. IC50 values of the synthesized compounds were calculated for each cell line following treatment for 72 h (Table 2). The high cytotoxicity found in all cancer cell types may address the possible role of these compounds on common cancer activated pathways.

In general, boehmeriasin A (1) and its hydroxy analog 19 (as 1:2 mixture of diastereomers) were identified as promising anti-cancer compounds due to their low nanomolar IC50 values. Although ketone derivative 13 showed moderate cytotoxicity, when compared to 1 and 19 its bioactivity was low despite the only minor alteration of its structure. Additionally, the carboxylate species 12 had no cytotoxic bioactivity against these cancer cells, possibly because it does not easily permeate into cells due to its ionic nature. Furthermore, this result is in line with the loss of activity seen for the other ‘open-structured’ analogs 15–18.
Considering the specific lack of any HCC targeted chemotherapeutic agents and based upon our specific aim in this study, we furthermore tested these compounds (1, 12, 13 and 19) against various liver cancer cell lines and normal transformed breast epithelial cells since there are no transformed normal liver hepatocytes available. Therefore, we assessed the IC50 values of these compounds in a HCC cell panel (Huh7, Mahlavu, SNU475, FOCUS, Hep3B and HepG2) (Table 3).

Similar to our results presented in Table 2, compounds 1 and 19 were found to be significantly cytotoxic in all cell types of HCCs. Crucially, 1 and 19 displayed nanomolar IC50 values not only for well differentiated HCC cells such as Huh7, Hep3B and HepG2 but also for poorly differentiated and more aggressive cells as tested here, namely, Mahlavu, FOCUS and SNU475. Furthermore, normal transformed epithelial cells were less sensitive to compounds 1 and 19. Our data showed significant cytotoxic effects of compounds in the nanomolar range in cancer cells while the cytotoxic effect on normal transformed cells was diminished (Table 3). The higher IC50 found in normal like HepG2 cells further supported the cancer-specific activity of these compounds. Therefore, 1 and 19 can be considered potential anti-cancer drug candidates in liver cancer cells. On the other hand, 13 displayed limited cytotoxic activity and 12 had no activity in any type of HCC cells (data not shown).

2.3. Real-time assessment of hepatocellular carcinoma cell growth upon treatment with compounds 1 and 19

A real-time cell electronic sensing (xCELLigence RTCA analysis) assay was used to evaluate the bioactivities of Boehmeriasin A and its analog 19 on well differentiated Huh7 cells and poorly differentiated aggressive mesenchymal Mahlavu cells (25). According to the data obtained from this analysis, the time dependent IC50 values were calculated (Table 4). Compounds 1 and 19 caused significant growth inhibition in both cells. Drug resistant Mahlavu
cells were prominently more sensitive to \textbf{19} (Fig. 3). The real-time growth inhibition pattern suggested a cell cycle arrest in cells treated with \textbf{1} and \textbf{19}, as DMSO treated cells retain their proliferation profile until they become confluent.

The curvature of the real time cell death data and the measured IC\textsubscript{50} values imply that the cell death associated with these compounds could be apoptosis. The differences of IC\textsubscript{50} values between SRB end point colorimetric assay and the real time cell growth assays can be due to the evaluation of distinct cellular elements. While SRB detects the total protein levels, RTCA is dependent on the cell surface attachment properties of each different cell. During apoptosis there is a high destruction of cellular proteins which leads to less colorimetric data collection. Therefore, it is common to observe IC\textsubscript{50} differences between cell death detection techniques. Hence in this study, we established the bioactivities of the new boehmeriasin A analogs using two complementary techniques.

### 2.4. Characterization of cell death mechanism induced by boehmeriasin derivatives

The cell death mechanism of the cells treated with boehmeriasin A or its derivatives was initially analyzed by observing the release of cytochrome-C which was examined via fluorescence microscopy (Fig. 4A). Camptothecin (CPT) was used as positive control for cytochrome-C release, showing that cytochrome-C levels in cells treated with \textbf{1} and \textbf{19} were comparable to the positive control. A significant increase in the release of cytochrome-C in cells treated with \textbf{1} and \textbf{19} indicates apoptotic cell death.

The activation of apoptotic pathways by boehmeriasin A derivatives was further studied by evaluating the status of one of the most well-known apoptotic proteins, PARP. Here a significant increase in PARP cleavage in the cells treated with \textbf{19} was identified and became significant within 72 h in cells. Compared to boehmeriasin A itself, the hydroxy-derivative \textbf{19} displayed a more significant effect on PARP cleavage that is paralleled by its increased cytotoxic effect on liver cancer cells (Fig. 4B).

During cellular apoptosis, many biochemical changes occur. Once the apoptosis mechanism is triggered DNA fragmentation, phosphatidylserine flipping to the outer surface of the cells and some morphological changes such as smaller cell size, chromatin condensation and formation of apoptotic bodies eventually occur. Hoechst is a fluorescence dye that attaches to DNA of both dead and living cells and makes their nucleus visible. The intensity of Hoechst staining (chromatin condensation), structure of nucleus and formation of apoptotic bodies are the initial indicators of cellular apoptosis. The Hoechst staining of \textbf{1} and \textbf{19} treated cells

### Table 4

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<tr>
<th>Compound</th>
<th>Huh7</th>
<th>Mahlavu</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>\textbf{1}</td>
<td>1.4 \textmu M</td>
<td>0.7 \textmu M</td>
</tr>
<tr>
<td>\textbf{19}</td>
<td>0.8 \textmu M</td>
<td>0.5 \textmu M</td>
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\textbf{Fig. 3.} xCELLigence RTCA analysis. Cell growth index with various concentrations of the compounds \textbf{1}, \textbf{19} and their corresponding DMSO solvent controls on Huh7 and Mahlavu cell lines.
showed many indicators of apoptosis (chromatin condensation and apoptotic body formation). The effect was increased at 72 h for all samples.

Moreover, the apoptosis of cells treated with 1 and 19 was further demonstrated with Annexin V assay via flow cytometry. Normally, phosphatidylserine (PS) residues are hidden in the membrane of living cells. Upon apoptosis, PS flips from the inner cell to the outer surface which then can be detected by Annexin V. This assay showed the sharp increase in apoptosis of cells treated with 1 and 19 for epithelial Huh7 cells at 72 h. The drug response was similar in Mahlavu cells at 72 h but 1 showed less dramatic effect on these cells. The Annexin V results thus supported the previous findings.

2.5. Cell cycle analysis of HCC cells treated with boehmeriasin derivatives

To identify the effect of the boehmeriasin derivatives on cell cycle progress, flow cytometry analysis was performed using propidium iodide (PI) staining in Huh7 and Mahlavu liver cancer cells. Upon treatment with compounds 1 and 19, increasing levels of apoptotic cells along with SubG1 arrest were observed. The most significant increase was observed at 72 h. Derivative 19 resulted in the higher number of cells that accumulated at SubG1 phase. Additionally, HCC cells treated with 1 also led to an increase in SubG1, however, this effect became more notable after 72 h (Fig. 5 A, B). In addition, the effect of boehmeriasin A derivatives on the cell cycle was further investigated in Huh7 and Mahlavu cells by western blot analysis of the principal proteins which are involved in this process. Compounds 1 and 19 down-regulated Cyclin B1 (CDK1 regulator and mitotic initiator in the late G2 phase) and its companion CDK1 (essential for G1/S and G2/M phase transitions) levels in Huh7 cells. Treatment with 1 and 19 also reduced the CDK2 (G1 to S phase transition) and its regulator Cyclin E level, which became more significant at 72 h. Combined, these results indicate that boehmeriasin A derivatives induce cell cycle arrest at G2/M and SubG1 phases which is then followed by apoptosis of the cells (Fig. 5A).

The effect of these compounds on cell cycle protein expression is more pronounced on Huh7 cells (Fig. 5B). Mahlavu cells are
reported as more resistant to small molecule inhibitors due to PTEN deletion in PI3K/Akt signaling pathway [25]. However, the effect of compounds 1 and 19 can still be observed even in these cells. Furthermore, the active phosphorylated form of Akt protein is significantly down-regulated with compound 19 at 72 h (Fig. 5B).

2.6. Release of reactive oxygen species and stress mechanism induced by boehmeriasin derivatives

Reactive oxygen species (ROS), depending on their dose, can alter cellular pathways and promote cell cycle arrest and apoptosis in liver cancer cells [26]. The released ROS can be visualized using the 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. The presence of fluorescence stain, which is detected via fluorescence microscopy, indicates ROS activity and thus cellular stress. Evaluating the presence of ROS in liver cancer cells that have been treated with boehmeriasin A and its derivatives showed that the ROS activity in cells incubated with 1 and 19 was significantly increased and became comparable to the positive control. The ROS activity increased with the time of incubation of 1 and 19 (Fig. 6A).
In addition, these Boehmeriasin derivatives induced oxidative stress, that was assessed by evaluating the phosphorylation status of the proteins involved in the ROS pathway via western blot [25]. It was found that Mahlavu and Huh7 cells treated with 1 and 19 led to a decrease of the phosphorylated Ser-966-ASK1 levels which is associated with cellular oxidative stress, though the effect on Mahlavu is being more prominent [25] (Fig. 6B). Mahlavu cells are characterized by a hyperactive PI3K/AKT pathway due to PTEN protein deletion and therefore we observe differential downstream P-SAPK/JNK protein levels. The stress protein SAPK/JNK phosphorylation was increased in Huh7 cells treated with both 1 and 19. In Mahlavu cells the P-SAPK/JNK levels were differentially altered.

Fig. 5. The assessment of the cell cycle status of cells treated with boehmeriasin A derivatives. (A) The time dependent cell cycle analysis of treated Huh7 and Mahlavu cells with flow cytometry. (B) Comparative analysis of cell cycle protein expression in the presence of 1 and 19. DMSO is used as the solvent control.
The compound 19 decreases the p-AKT levels which is the active form of this protein required for cell survival. Our data indicates that the novel compound 19 gains its intriguing bioactivity through PI3K/AKT pathway modulation which is strategically involved in liver cancer tumorigenesis.

Fig. 6. The analysis of cellular stress mechanism. (A) The evaluation of ROS activity by DCHF-DA staining in Huh7 cells treated with boehmeriasin derivatives. DCFH-DA is a cell permeable dye, and, after uptake, it is cleaved by intracellular esterases to 2,7-dichlorodihydrofluorescein (DCFH2), which is trapped within the cells, and is oxidized to fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. The cells that grow in serum free medium were shown to induce ROS activity, therefore, we used serum free culture media as positive control. (B) The western blot analysis of the stress related proteins of boehmeriasin derivatives treated Huh7 and Mahlavu cells.

2.7. The bioactivity of individual hydroxy-analogs 19a and 19b on epithelial cancer and normal epithelial immortalized cells

Having determined that 19 (originally tested as a 1:2 mixture of diastereomers 19a and 19b) possessed the highest bioactivity, we decided to further study its individual diastereomers to establish...
whether a preference of the relative stereochemistry manifests in differentiated activity. The separation of this mixture of diastereomers was accomplished by preparative thin layer chromatography yielding 19a as the minor (lower Rf) and 19b as the major (higher Rf) diastereomer in a 1:2 ratio. The individual hydroxy-analogs 19a and 19b were initially screened in human liver, colon and breast cancer cells along with immortalized transformed normal breast epithelial cells (Table 5). The data was verified with SRB results confirming not only that both hydroxy-analogs were highly active anti-cancer compounds but also 19b displayed superior activity in the nanomolar range.

Correlating this intriguing finding with the single crystal X-ray structure secured for the major diastereomer 19b (see Scheme 3) demonstrates that the highest activity results from the diastereomer having the hydroxy substituent trans to the methine proton and syn to the ring nitrogen. Although, the reason of this is not entirely clear at this point this finding might point to a specific binding mode of 19b within its molecular target.

2.8. The induced apoptosis of individual hydroxy-analogs 19a and 19b

After we showed the cytotoxicity of 19a and 19b in nanomolar range, we further studied the cell death mechanism of these compounds in Huh7 and Mahlavu cells. Initially, treated cells were stained with Hoechst dye and their apoptotic morphologies were analyzed. The Hoechst staining of 19a and 19b treated cells showed a high number of apoptotic cells which possessed condensed chromatin and apoptotic bodies, compared to the DMSO control. The effect was increased for 19b treated Huh7 cells which showed more apoptotic features compared to 19a at 72 h. The apoptotic effect of compounds was similar in Mahlavu cells. A reduced number of remaining cells was observed after 19b treatment in Mahlavu cells, therefore, the percentage of apoptotic cells had increased (Fig. 7A).

Table 5

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<thead>
<tr>
<th>Compound</th>
<th>IC50 Values (µM)</th>
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<tr>
<td></td>
<td>Huh7</td>
</tr>
<tr>
<td>19a (minor)</td>
<td>0.306</td>
</tr>
<tr>
<td>19b (major)</td>
<td>0.017</td>
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Fig. 7. The analysis of apoptosis. (A) The nucleus staining with Hoechst dye (blue) to identify the cellular apoptosis. White arrows indicate the apoptotic cells. (B) The Annexin V analysis of Huh7 and Mahlavu cells treated with 1 µM 19a and 19b for 24, 48 and 72 h. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Furthermore, the Annexin V assay was utilized to analyze the apoptotic effect of 19a and 19b in more detail. Annexin V results showed the significant increase of apoptotic cells in 19b treated cells, especially at 72 h. Similar to previous results, this assay further demonstrated the major compound 19b had a severe effect on cellular apoptosis when compared to 19a (Fig. 7B). Together these results supported the findings of SRB assay which revealed their high cytotoxicity.

In conclusion, we have successfully prepared two sets of new boehmeriasin A analogs featuring either a simplified structure or a complete pentacyclic framework as found in the parent alkaloid. Whilst the former was found to be significantly less active (IC50 > 10 µM) than boehmeriasin A, the latter series displayed superb anti-cancer activity across a range of cell lines. Minute structural changes from a ketone (13, H-bond acceptor) to a secondary alcohol (19, H-bond donor) have resulted in a 10,000-fold increase in activity, which is more pronounced in the trans-diastereomer (19b) and surpasses the activity of boehmeriasin A itself. Subsequent in-depth biological studies that were performed for the first time in HCC cells (well differentiated Huh7 and poorly differentiated aggressive Mahlavu cells) and have revealed a broad spectrum of activities including cell cycle arrest in the sub-G1 stadium, the generation of reactive oxygen species as well as the activation of apoptotic pathways through PARP cleavage. Moreover, Annexin V and Hoechst staining results further demonstrated the induction of apoptosis in drug treated cells. Due to the straightforward synthetic accessibility and the intriguing biological properties of our new lead compound 19b we foresee its potential for future developments to address unmet challenges within liver cancer therapy.

3. Experimental section

3.1. Synthesis procedures for new compounds

Purity of all compounds was established via HPLC using an Agilent 1100 instrument with a Zorbax C18 column (4.6 x 100 mm) and UV-detection at 220 nm. An isotropic eluent system was used (60:40 MeCN:water, containing 0.1% TFA; 1 mL/min). All compounds displayed purities above 95% unless stated otherwise.

Rac-Boehmeriasin A, 1 and 3,6,7-trimethoxy-12,13,14-tetrahydro-9H-dibenzo[f,h]-pyrido[1,2-b]isoquinolin-15(11H)-one, 13 were prepared as reported previously [24] and displayed purities of 97% and 96% respectively by HP-LC.

(E)-N-(3-(11H-imidazol-1-yl)propyl)-2-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)acryl-amide, 15: To a
solution of methyl (E)-2-(3,4-dimethoxyphenyl)-3-(4-
methoxyphenyl)acrylate (8, 1.0 g, 3.05 mmol) in methanol (10 mL, 0.3 M) was added an aqueous solution of KOH (5.0 M, 5 mL, 8.3 equiv. KOH). The resulting mixture was stirred at 50 °C for 4 h when TLC analysis indicated full conversion of 8. After neutralizing with aqueous HCl (1 M) and extraction (DCM/water, 2 × 50 mL) the organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure. The solid residue was dissolved in MeCN (15 mL) and combined with carboxydimidazole (CDI, 500 mg, 3.09 mmol, 1 equiv.). After heating this mixture for 1 h at 50 °C amipropinomimidazole (275 mg, 3.00 mmol, 1 equiv.) was added and the mixture was stirred at 50 °C for a further 2 h. After evaporation of the volatiles the crude product was extracted (DCM/water, 2 × 20 mL) and isolated as an off-white solid (1.0 g, 2.4 mmol, 78%) after drying over anhydrous sodium sulfate, filtration, evaporation, and purification from cold ethyl acetate.

1H-NMR (400 MHz, CDCl3): δ/ppm 7.68 (s, 1H), 7.34 (d, J = 1.2 Hz, 1H), 6.92 (d, J = 1.1 Hz, 1H), 6.91–6.87 (m, 3H), 6.83 (d, J = 1.3 Hz, 1H), 6.72 (dd, J = 8.2, 1.9 Hz, 1H), 6.64 (d, J = 1.9 Hz, 1H), 6.60 (d, J = 8.9 Hz, 2H), 5.73 (t, J = 6.1 Hz, 1H), 3.89 (t, J = 2.8 Hz, 2H), 3.86 (s, 3H), 3.72 (s, 3H), 3.66 (s, 3H), 3.23 (app q, J = 6.7 Hz, 2H), 1.50 (app q, J = 6.9 Hz, 2H), 1.53 (app q, J = 6.8 Hz, 2H). 13C-NMR (100 MHz, CDCl3): δ/ppm 167.2 (C), 158.9 (C), 149.9 (C), 149.1 (C), 137.0 (CH), 136.8 (CH), 132.0 (2CH), 131.3 (C), 129.5 (CH), 128.5 (C), 127.4 (C), 122.0 (CH), 118.8 (CH), 113.7 (2CH), 112.6 (CH), 112.2 (CH), 56.0 (CH2), 55.9 (CH2), 55.1 (CH2), 44.5 (CH2), 37.2 (CH2), 31.3 (CH3). IR (neat, ν/cm−1): 3375 (w), 2981 (s), 1655 (m), 1602 (m), 1509 (s), 1446 (m), 1252 (s), 1716 (m), 1139 (s), 1080 (m), 1019 (m), 959 (s), 833 (s), 738 (m), 620 (m). HR-MS (TOF-AP+) calculated for C32H42N4O4 422.2080, found 422.2079. HP- LC (220 nm) Rt = 1.72 min (97% purity).

N-(3-(1H-imidazol-1-yl)propyl)-2,3,7-trimethoxyphenanthrene-9-carboxamide, 16: In analogy to the preparation of 15, methyl 3,6,7-trimethoxyphenanthrene-9-carboxylate (9, 700 mg, 2.15 mmol) was converted into the target compound 16 (730 mg, 1.74 mmol, 81%) which was obtained as a yellow waxy solid.

1H-NMR (400 MHz, CDCl3): δ/ppm 7.71 (s, 1H), 7.65 (s, 1H), 7.63 (d, J = 2.5 Hz, 1H), 7.61 (d, J = 8.8 Hz, 1H), 7.50 (s, 1H), 7.49 (s, 2H), 7.10 (dd, J = 8.7, 2.4 Hz, 1H), 7.06 (t, J = 5.9, 5.9 Hz, 1H), 6.99 (t, J = 1.1 Hz, 1H), 6.93 (t, J = 1.3 Hz, 1H), 4.03 (s, 4H), 4.01 (t, J = 1.8 Hz, 2H), 3.97 (s, 3H), 3.92 (s, 3H), 3.43 (app q, J = 6.8 Hz, 2H), 2.07 (p, J = 6.8 Hz, 2H). 13C-NMR (100 MHz, CDCl3): δ/ppm 170.5 (C), 159.2 (C), 149.6 (C), 149.1 (C), 137.2 (CH), 131.9 (C), 130.7 (CH), 129.4 (CH), 129.1 (C), 123.4 (CH), 124.7 (CH), 124.2 (C), 124.1 (C), 118.9 (CH), 115.9 (CH), 106.4 (CH), 103.7 (CH), 103.2 (CH), 55.9 (CH2), 55.8 (CH2), 55.5 (CH3), 44.8 (CH2), 37.2 (CH2), 31.2 (CH2). IR (neat, ν/cm−1): 3259 (broad), 2939 (m), 1618 (m), 1522 (s), 1508 (s), 1473 (s), 1270 (s), 1228 (s), 1206 (s), 1158 (m), 1033 (m), 832 (w), 734 (w). HR-MS (TOF-AP+) calculated for C14H18N2O2 240.1923, found 240.1921. HP- LC (220 nm) Rt = 1.64 min (98% purity).

(E)-N-(3-(1H-imidazol-1-yl)propyl)-2-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-amine, 17: To a solution of E-N-(3-(1H-imidazol-1-yl)propyl)-2-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)acrylamide (15, 500 mg, 1.19 mmol) in dry THF (10 mL, 0.12 M) was added lithium aluminum hydride (LAH, 200 mg, 5.26 mmol) in portions at room temperature. After 2 h the reaction mixture was carefully quenched by addition of ethyl acetate (1 mL) and water (3 mL). The resulting emulsion was filtered through a pad of celite (ca. 10 g) and washed with ethyl acetate (30 mL). The resulting pale-yellow solution was evaporated to dryness yielding a yellow oil that was purified by column chromatography on silica (20–40% EtOAc/hexanes). The desired product 17 was obtained as a colorless oil that solidified upon standing (445 mg, 1.09 mmol, 92%).
3.8 Hz, 1H), 2.07 (d, J = 13.1 Hz, 1H), 2.24 (qd, J = 13.3, 3.8 Hz, 1H), 2.07 (d, J = 11.2 Hz, 1H), 1.90–2.00 (m, 2H), 1.82 (q, J = 13.1 Hz, 1H), 1.73 (d, J = 13.1, 1H), 1.67 (d, J = 12.2 Hz, 1H), 1.27–1.35 (m, 1H). 13C-NMR (151 MHz, CDCl3); δ/ppm 157.4 (C), 148.5 (C), 148.3 (C), 130.4 (C), 127.8 (C), 126.8 (CH), 124.7 (C), 124.0 (C), 123.5 (2C), 111.4 (CH), 104.0 (CH), 102.9 (CH), 102.5 (CH), 66.5 (CH), 62.5 (CH), 56.7 (CH2), 55.6 (CH2), 55.4 (CH3), 55.3 (CH3), 55.2 (CH2), 27.0 (CH2), 25.1 (CH2), 24.1 (CH2). IR (neat, v/cm−1): 3184 (broad), 2938 (m), 1743 (w), 1611 (m), 1534 (m), 1511 (s), 1470 (s), 1424 (m), 1257 (s), 1204 (s), 1171 (s), 1127 (m), 1042 (s), 910 (m), 839 (m), 730 (s). HR-MS (ESI-AP+) calculated for C24H28NO4: m/z 394.2018, found 394.1960. HP-LC (220 nm) Rt = 1.77 min (96% purity). Crystal data for 19b: C39H39Cl3N4O4, M = 748.39, orthorhombic, space group Pbca, a = 12.2463(8), b = 25.3267(16), c = 29.2135(18) Å, U = 9060.8(10) Å³, F(000) = 4032, Z = 16, Dc = 1.403 mg m⁻³, μ = 0.320 mm⁻¹ (Mo-Kα, λ = 0.7073 Å), T = 120(1) K. Crystallographic data for the structure have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC-1842540.

3.2. Biological evaluation

3.2.1. Cells culture

Hepatocellular carcinoma cell lines (HuH7, FOCUS, Hep3B, HepG2 and Mahlavu), human breast cancer cells (MCF7) and human colon carcinoma cells (HCT116) were grown in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO), 1% non-essential amino acids (GIBCO, Invitrogen), 1% L-glutamine (GIBCO, Invitrogen, NY). All media composed with 1% (v/v) trichloroacetic acid (MERCK) for an hour. The solutions were stained with sulforhodamine B solution (50 μg/mL) for 10 min. The excess amount of SRB dye was discarded after 30 min interval. After 24 h of incubation, the cellular growth inhibition was calculated based on the Bradford assay. 25–50 μg from all proteins were loaded to Bis-Tris gel and western blot was performed with Novex NuPAGE Bis-Tris Electrophoresis system. The proteins were transferred to nitrocellulose membrane via XCell IEF Blot Module. Cyclin-B1 (554.177, BD), Cdc2/Cdk1 (PC2S, Calbiochem), PARP (9532, Cell Signaling), Cyclin E (CC05, Calbiochem), Cdk2 (sc52438, Santa Cruz), phosphor ASK1 (3765, Cell Signaling), phosphor JNK (9251, Cell Signaling), phosphor AKT (9275, Cell Signaling) and AKT (9272, Cell Signaling) antibodies were used in 1:100 to 1:500 BSA-TBS-T, β-actin (#A54541, Sigma) and Calnexin (C4731, Sigma) antibodies were used in 1:1000 concentration for equal loading.

3.2.6. Immunofluorescence assay

HuH7 and Mahlavu cells were seeded into 6 well plates on coverslips. After 24 h, cells were treated with the 1 μM Boehleriasin derivatives for 72 h. Camptothecin was used as positive control for cytotoxicity activity. DMSO control was given to cells in the same amount of the compounds. After the incubation period, cells were fixed with ice-cold methanol for 15 min. The cytokeratin primary antibody (Santa Cruz, 1:100 in 0.1% TBS-Tween) were applied for 1 h. FITC conjugated secondary antibody (Santa Cruz, 1:200 in 0.1% TBS-Tween) were applied for 1 h. The cells were mounted with UltraCruz DAPI mounting medium and photos were taken with fluorescence microscope.

3.2.7. Flow cytometry for cell cycle analysis

HuH7 and Mahlavu cells were seeded onto 100 mm culture dishes. After 24 h, cells were treated with the 1 μM Boehleriasin derivatives. After 24 h, 48 h and 72 h of incubation, cells were fixed with ice-cold 70% ethanol for 3 h at −20 °C. Cell cycle analysis was carried out by PI (Propidium iodide) staining using MUSE Cell Analyzer according to the manufacturer’s recommendations (Millipore).

3.2.8. Flow cytometry for annexin

30.000 Mahlavu and 60.000 HuH7 cells were plated to 6 wells. After 24 h, cells were treated with 1 μM of 1, 19a and 19b. The cells were collected with trypsin after 24, 48 and 72 h of treatment. The supernatants were also collected into same tube. The cells were centrifuged at 1600 rpm for 6 min and 100 μL of annexin stain solution was added on top of the pellets. The cells were incubated for 15 min with stain solution in the dark. Before analyzing the samples, 400 μL 1xPBS was added and cells were resuspended. The Annexin V samples were analyzed with a Novocye Flow Instrument.

Annexin stain solution (Roche-11988549001): 2 μL Annexin-V-FLUOS labeling reagent and 2 μL propidium iodide solution were added in 100 μL incubation buffer per sample.
3.2.9. Hoechst nuclear staining

30.000 Mahlavu and 60.000 HuH7 cells were plated to 6 wells on to coverslips. After 24 h, cells were treated with 1 μM of 1, 19a and 19b. The cells were fixed with ice-cold ethanol after 24 and 72 h of treatment. Samples were washed with 1X PBS three times and samples were incubated via Hoechst staining solution for 10 min. Samples were washed with distilled water and mounted with glycerol. Hoechst staining was analyzed via fluorescence microscopy (Nikon).

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.01.056.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CCR2</td>
<td>CC chemokine receptor 2</td>
</tr>
<tr>
<td>CCL2</td>
<td>CC chemokine ligand 2</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC chemokine receptor 5</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>CDI</td>
<td>carbonyl diimidazole</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>LAH</td>
<td>lithium aluminum hydride</td>
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References