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Design, Synthesis and Biological Study of Hybrid Drug Candidates of Nitric Oxide Releasing Cucurbitacin-Inspired Estrone Analogs for Treatment of Hepatocellular Carcinoma

Mahrous A. Abou-Salim a,d, Mohamed A. Shaaban b, Mohammed K. Abd El Hameid b, Yaseen A.M.M. Elshaier c, Fathi Halaweish d,*

a Al-Azhar University, Faculty of Pharmacy, Pharmaceutical Organic Chemistry, Assiut 71524, Egypt
b Cairo University, Faculty of Pharmacy, Pharmaceutical Organic Chemistry, Cairo 11562, Egypt
c University of Sadat City, Faculty of Pharmacy, Organic and Medicinal Chemistry, Menoufia 32958, Egypt
d South Dakota State University, Chemistry & Biochemistry, Box 2202, Brookings, SD 57007, USA

Abstract

Development of hybrid drug candidates is well known strategy for designing antitumor agents. Herein, a novel class of nitric oxide donating cucurbitacin inspired estrone analogs (NO-CIEAs) were designed and synthesized as multitarget agents. Synthesized analogs were initially evaluated for their anti-hepatocellular carcinoma activities. Among the tested analogs, NO-CIEAs 17 and 20a exhibited more potent activity against HepG2 cells (IC₅₀ = 4.69 and 12.5 µM, respectively) than the reference drug Erlotinib (IC₅₀ = 25 µM). Interestingly, NO-CIEA 17 exerted also a high potent activity against Erlotinib-resistant HepG2 cell line (HepG2-R) (IC₅₀ = 8.21 µM) giving insight about its importance in drug resistance therapy. Intracellular measurements of NO revealed that NO-CIEAs 17 and 20a showed a significant increase in NO production in tumor cells after 1h of incubation comparable to the reference prodrug JS-K. Flow cytometric analysis showed that both NO-CIEAs 17 and 20a mainly arrested the HepG2 cells in the G0/G1 phase. Also, In-Cell Based ELISA screening showed that NO-CIEA 17 resulted in a potential inhibitory activity towards the EGFR and MAPK (25% and 29% inhibition compared to untreated control cells, respectively). This data suggests the binding ability of NO-CIEA 17 to the EGFR and ERK to be well correlated along with the docking and cellular studies. Also, treatment of HepG2-R cells with NO-CIEA 17 showed a potential reduction of MRP2 expression in a dose dependent manner providing a significant impact on the chemotherapeutic resistance. Overall, the current study provides a potential new approach for the discovery of a novel antitumor agent against HCC.
Keywords

Nitric oxide; HCC; EGFR; DAF-FM DA; MRP2; MAPK; NO-CIEAs

1 Introduction

Hepatocellular Carcinoma (HCC) is the most common primary malignancy of the liver and the third most fatal cancer worldwide since it has high incidence to mortality ratio (1.07) [1, 2]. Among all cancers, HCC is one of the fast growing causes of death in the US with a death to survival ratio of 0.72 in 2018 [3, 4]. In Egypt, HCC is the second most common cancer in men and the sixth most common cancer in women [5]. It poses a significant economic burden on healthcare [4]. HCC has a multitude of etiological risk factors that have also been associated strongly with its development such as hepatotropic viruses (HBV, HCV, and hepatitis D virus (HDV)), alcoholism, aflatoxin, cirrhosis, autoimmune hepatitis, nonalcoholic steatohepatitis (NASH), hemochromatosis (Iron overload in the body), and type 2 diabetes (probably aided by obesity) [4].

For patients with advanced stage HCC, systemic therapy with epidermal growth factor receptor specific tyrosine kinase inhibitors (EGFR-TKIs) such as Erlotinib (Tarceva®) (Fig. 1) to induce cell cycle arrest and apoptosis and increase chemo sensitivity is recommended [6]. The major problems associated with the available chemotherapeutic agents are drug resistance, which occurs with 30% - 80% of cancer patients. HCC cells produce drug resistance via three mechanisms: multidrug resistance (MDR), P-glycoprotein drug resistance (P-gp) and multidrug resistance protein (MRP). All of them are activated and effective in the HCC treatment process [7, 8]. Therefore, there is an urgent need to design a new active molecular target anti-cancer agent for HCC to overcome the toxicity and cellular resistance problems.

Nitric oxide (NO) is an endogenous short-lived free radical obtained from conversion of L-arginine to L-citroline [9], which is mediated by nitric oxide synthase (NOS) [10]. NO producing compounds have a wide biological function as anticancer agents in addition to their gastrointestinal safety and hepatoprotective properties [9, 11-15]. It is also known to reverse chemotherapy resistance in cancer as colon cancer and inhibited the MRP3 efflux pump by nitrating the tyrosine and thereby led to inversion of drug resistance [16-18]. Hence, it is likely
that NO can be used in conjunction with traditional cancer drugs that have become ineffective due to such modes of efflux. It is noteworthy to mention that, NCX-1000 (Fig. 1) is the first hepatoprotective NO donor, however, it has not met the endpoint in phase 2 clinical trials [11, 19-21].

Cucurbitacin’s (CUCs) is highly oxidized tetracyclic triterpenoids isolated from a well-known anticancer family Cucurbitaceae. Previous studies on natural or semi-synthetic CUCs in our research group and other scientists demonstrated the ability of several types of CUCs to target EGFR-TK and the whole MAPK signaling pathway using mutant B-Raf cell lines through inhibition of proliferation and induction of apoptosis [22-24]. Among them, cucurbitacin D, E and I (Fig. 1) had a potent in vitro cytotoxic activity against HepG2 cell line and prolonged the survival time, life span and normalizes the biochemical parameters of mice-bearing tumor of Ehrlich’s ascites carcinoma [22, 25]. CUCs are formed in minute quantities in plants and isolating substantial amounts remain a challenge. Also, the existing methodology available for the total synthesis of these compounds would not be sufficient for the procurement of sufficient quantities for detailed biological evaluation or the synthesis of analogues. Interestingly, CUCs possess an estrone bioisosteric base-scaffold (Fig. 2) and this devoted us to in silico design and synthesize estrone analogs-hybrid of CUCs side chain and estrone base-scaffold against EGFR kinase domain [26].

Recently, Korita et al [27] suggested that efficacy of cisplatin-based chemotherapy in patients with HCC depends upon MRP2 expression level [27]. MRP2 is the second member of the MRP subfamily of ABC transporter. The first time MRP2 was cloned from rat hepatocyte and was named as a hepatocellular canalicular multiple organic anion transporter (cMOAT) [28]. In addition, MRP2 shares 49% amino acid identity with MRP1 but it has a different expression pattern. While MRP1 is widely expressed in many tissues, MRP2 is mainly expressed in the apical (canalicular) hepatocyte plasma membrane, small intestine, and renal proximal tubules [29]. Because of the similarity between MRP2 and MRP1, it was believed to confer resistance to similar anticancer drugs too. This hypothesis was created based on an experiment in which an antisense RNA construct was introduced into human HCC HepG2 cells, causing in increased sensitivity to several anticancer drugs such as cisplatin, vinblastine, sorafenib, doxorubicin, and erlotinib [28]. MRP2 has been demonstrated to transport vinblastine in polarized Madin Darby canine kidney epithelial (MDCK) cells, proposing a potential role for MRP2 in resistance. In addition, transfected cells also conferred resistance to cisplatin, etoposide, doxorubicin, and epirubicin. This phenomenon is convincible to suggest a potential role for MRP2 in drug resistance such as erlotinib against HepG2 resistance cell line [30, 31].
Enlightened by the aforementioned and on continuation to our previous work [32], we envisioned that tethering NO releasing moiety to CIEAs known of potent inhibition of HCC, will significantly impact the potential anticancer activity of hybrid drug candidate as a result of synergistic effects of NO and CIEA. In this context, we aim to design and synthesize novel hybrid drug candidates of nitric oxide releasing cucurbitacin-inspired estrone analogs (NO-CIEAs) as potential nitric oxide releasing motifs targeting EGFR-ERK signaling pathway for treatment of HCC and overcoming the chemotherapeutic resistance.

2 Design strategy

Modification of ring A of estrone has received substantial attention to block the estrogenic activity of the free phenolic OH group of estrone analogs [33]. So, we envisioned that tethering the C-3 phenolic OH with NO-releasing functionality is expected to have a significant impact on the anticancer activity of CIEAs with dual effect; block the estrogenic effect and site of NO source. Furoxan and organic nitrate moieties were chosen as NO donor source (Fig. 2).

Also, incorporation of various pharmacophores at C-17 of ring D has produced analogs that induce apoptosis in different cancer cell lines [23, 24]. Therefore, to investigate further biological activity associated with the C-17 functionalized estrone structure and its side chain, a new set of estrogen derived targets were identified for synthesis of novel hybrid molecules with high binding affinity to molecular targets (Fig. 2).

In this context, we envisioned that a novel drug candidate of hybrid structure 1) the NO donating group appendix on phenolic OH; 2) using estrone base-scaffold as it considered as natural bioisostere to CUCs architecture and finally 3) C-17 functionalization with important CUCs side chain as well as aryl and heterocyclic substituted α,β-unsaturated ketones assembled to CUCs side chain. The chemical structures were chosen and constructed by utilizing molecular docking for many runs before starting synthetic study. All designed compounds were fully characterized and underwent biological investigations to evaluate their activity for treatment of HCC. Also, this widens our armaments against a devastating disease that we are failing to face.
3 Results and Discussion

3.1 In silico molecular modeling

A library of NO-CIEA hybrids was designed and energy minimized using MMFF94 force field calculations. The catalytic domain of two different receptors EGFR (PDB code: 1M17 [34]) and ERK (PDB code: 2OJJ [35]) were downloaded from PDB and prepared for docking using Open Eye® software [34]. Different conformations of each ligand were generated by Open Eye® Omega application. Docking was conducted using Fred® and the data was visualized by Vida® application. Consensus scoring which is a filtering processes to obtain virtual binding affinity will be generated and the lower consensus score, the better binding affinity of the ligands towards the molecular targets. Docking study was operated with multi running time and the best 10 compounds were selected to the next run and then 50-70 compounds were derivatized a library component.

The docking study with EGFR revealed that all the target compounds showed better consensus score than the lead drug Erlotinib, NCX-1000 and CUCs D, E and I, Table 1. To validate our docking method, the standard Erlotinib showed a hydrogen bond (HB = 2.27 Å) towards ATP-binding site of EGFR coming from pyrimidine-N2 with Met:769:A (Fig. 3A). This docking mode and pose similar to co-crystalized docking pose of Erlotinib with receptor [34]. NO-CIEAs 17 (Fig. 3B), 20a,b,g, 23a,b, 30 and NCX-1000 showed only a hydrophobic-hydrophobic interaction towards the ATP binding site of EGFR. Also, NO-CIEA 20f showed a hydrophobic-hydrophobic interaction towards the ATP binding site as well as a HB between C-19 hydroxyl group (donor) and carbonyl oxygen (acceptor) of Met:769:A with bond length 2.30 Å (Fig. 3C). Furthermore, 20d and 20e showed a hydrophobic-hydrophobic interaction towards the ATP binding site as well as a hydrogen bond between Lys:721:A (donor) and carbonyl oxygen of ring D of 20d (acceptor) and furoxan oxygen of 20e (acceptor) with bond length 1.56 and 2.11 Å, respectively, while compound 20c showed a hydrogen bonding between C-19 hydroxyl group (donor) and carbonyl oxygen of Asn:818:A (acceptor) with bond length 1.99 Å. Moreover, as shown in Fig. 3D, there is an overlay of the docking pose of NO-CIEAs 17 (gray) and 20f (green) in the EGFR active site.
Then, the docking study with ERK kinase domain (PDB code: 2OJJ) [35] revealed that NO-CIEA 17 shows a consensus score better than the co-crystallized ligand (82A; drug bank ID: DB07264) [35]. Table 1. The amide carbonyl of the co-crystallized ligand (82A) formed a HB with Lys:52:A. Its pyrazole-linked phenyl showed a hydrophobic interaction with Val:37:A of the glycine-rich loop. Also, the pyrrole NH showed a hydrogen bond with the side chain carbonyl of the gatekeeper residue Gln:103:A (Fig. 4A). This docking mode similar to co-crystalized docking pose with receptor [35]. The data also showed that the furoxan N2-oxygen of NO-CIEA 17 formed a HB (acceptor) with the Lys:52 (donor) (2.41 Å), and the steroidal backbone made a hydrophobic interaction with Val:37 of the glycine-rich loop. Also, the furoxan N4 was involved in a HB (acceptor) with the phenolic OH of Tyr:34:A (donor) with bond length 2.07 Å (Fig. 4B).

3.2 Chemistry

The synthetic pathway of the key intermediate α-hydroxy methyl ketones (7a,b) is summarized in Scheme 1. To install the C-17 side chain, the synthetic route was started with protection of the C-3 hydroxyl group of commercially available estrone 1 as a tert-butyldimethylsilyl (TBS) ether, followed by Wittig reaction with the ketone on ring D to prepare alkene 3 [36]. Hydroxylation of C-19 through hydroboration oxidation with 9-borabicyclo[3.3.1]nonane (9-BBN) upon heating to 60 °C in THF generated the alcohol 4 which was oxidized by PCC into methyl ketone 5 in 92% yield.

Treatment of methyl ketone 5 with catalytic amount of zinc iodide and TMSCN formed the desired nitrile 6 in 90% yield, as diastereomer mixture. The two diastereomers α-hydroxy methyl ketones 7a (19R-isomer) and 7b (19S-isomer) were successfully obtained by nucleophilic addition of methyl lithium to nitrile 6 in dry ether at 0 °C. The diastereomeric mixture was separated by careful column chromatography as they appeared as overlapped spots with ∆Rf less than 0.1 (5 % ethyl acetate in hexane). The absolute configuration was determined by NMR and this result is similar to the previous data [26, 37]. The 19R-isomer is the only isomer that was previously isolated and fully characterized. Herein we report the structural elucidation of 19S-
isomer by $^1$H NMR, $^{13}$C NMR, $^{13}$C DEPT-NMR and HSQC-NMR. The $^1$H NMR of S-isomer showed up-field chemical shift for some signals compared to R-one. Among them, the most important hydroxyl proton at C-19 which appeared at 3.49 ppm in case of S-isomer while in R-isomer it appeared at 3.80 ppm.

In order to install the desired CUCs side chain at C-17, the precursor 11 was synthesized as outlined in Schemes 2. Protection of 2-hydroxy isobutyrate 8 by TBSCl followed by reduction of ester functionality with DIBAL-H to produce primary alcohol 9. Then, controlled oxidation with either TPAP (Tetrapropylammonium perruthenate) or PCC yielded the protected aldehyde derivative 11.

On the other hand, to install the NO donor functionality, the furoxan precursor 14 was synthesized as outlined in Scheme 3. Under nitrogen atmosphere, cinnamyl alcohol 12 reacted with sodium nitrite in acetic acid to give 3-(hydroxymethyl)-4-phenyl-1,2,5-oxadiazole 2-oxide 13 which was converted to the corresponding mesylate derivative 14 [38, 39].

This set the stage for diverging the syntheses of the estrone analogs by reacting methyl ketone 7a with an alkyl, aromatic and heterocyclic aldehyde through an aldol condensation to provide the desired $\alpha,\beta$-unsaturated ketones. The target compound 17 was synthesized as outlined in Scheme 4 through installing the CUCs side chain at C-17 position then adding the NO donor moiety at the phenolic OH. Optimized reaction conditions in the aldol reaction of ketone 7a and aliphatic aldehyde 11 required the use of LDA (Lithium diisopropylamide) as a base in THF at -78 °C to produce acyclic $\alpha,\beta$-unsaturated ketone 15 in 48% yield. With material in hand, the deprotection of $\alpha,\beta$-unsaturated ketone 15 was attempted using tetra-n-butylammonium fluoride (TBAF) to afford phenolic derivative 16. Finally, the furoxan moiety was installed successfully to construct NO-CIEA 17 as depicted in Scheme 4.

Our direction was then oriented to add different aryl aldehydes to examine the effect of aromaticity in CUCs side chain. Aldol condensation of aromatic or heterocyclic aldehydes through enolate formation using sodium hydroxide, Scheme 5, was determined to be optimal here compared to the LDA procedure previously described in Scheme 4. This reaction condition resulted in a greater percent conversion, a higher yield, very short reaction time and a much more economical reagent. Deprotection of acyclic $\alpha,\beta$-unsaturated ketones 18a-g were subjected to a
similar TBAF procedure as before to give the phenolic derivatives 19a-g which reacted finally with furoxan mesylate 14 to give the target NO-CIEA 20a-g in good yields.

To explore the effect of absolute configuration around C-19 on compounds` biological activity, (19S)-NO-CIEAs 23a, and 23b with either strong electron withdrawing group (CF₃) or electron donating group (OCH₃), respectively, were prepared. Aldol condensation of 7b, with appropriate benzaldehyde derivative, using sodium hydroxide in THF method afforded acyclic α,β-unsaturated ketones 21a,b which were subjected to TBAF to obtain phenolic derivatives 22a,b. Finally, coupling them with furoxan mesylate 14 to get the desired (19S)-NO-CIEAs 23a,b in yields 39 and 34%, respectively as depicted in Scheme 6.

For further investigation of SAR, the intermediate 25 was designed and synthesized to study the effect of tethering the estrone base-scaffold with NO donor functionality without CUCs side chain. It was synthesized through deprotection of 7a with TBAF in THF overnight followed by coupling with furoxan mesylate 14 as outlined in Scheme 7. Moreover, to explore the effect of NO concentration on the anticancer activity, replacement of furoxan moiety with organic dinitrate ester at C-3 position of estrone was designed and synthesized as outlined in Schemes 7. Initially, the phenolic derivative 24 was subjected to O-allylation with allyl bromide to generate the O-allyl derivative 26. Addition of I₂ (1-3 eq.) to the intermediate 26 at different temperatures failed to synthesize the corresponding diiodo-derivative but in combination with silver nitrate (3 equivalents), the iodonitrate 27 was successfully synthesized in 86% yield as diastereomeric mixture in the ratio 1:1 (based on quantitative NMR detection). Next, refluxing the intermediate 27 with 3 more equivalents of silver nitrate resulted in 37% of compound 28 as diastereomeric mixture in the ratio of 1:0.5. Finally, the aldol condensation with the selected benzaldehyde derivative (p-CF₃PhCHO) in presence of NaOH for 10 min. afforded rapidly the target nitrate/CIE hybrid 29.

Next, our SAR study was extended to prepare the designed analogs 30 and 32 as starting materials for installing the organic dinitrate moiety at C-3 position along with CUCs and p-methoxy phenyl side chains at C-17 position, respectively. As shown in Scheme 8, because of the volatility of the aliphatic aldehyde 11, LDA procedure was used but the reaction proceeded with elimination forming a mixture of compounds 26 and 31. Their structures were elucidated by
\[ ^1H \text{ NMR, } ^{13}C \text{ NMR, } ^{13}C \text{ DEPT-NMR and HSQC-NMR. } \]

On the other hand, upon aldol condensation of 27 with \( p \)-methoxybenzaldehyde using NaOH in dry THF for 10 min., the reaction did not proceed. But after refluxing for 1 h, base-mediated cleavage occurred producing the undesired corresponding phenols 24 and 19d [40]. Structural elucidations of all separated products were verified by \(^1H \text{ NMR, } ^{13}C \text{ NMR and } ^{13}C \text{ DEPT-NMR} [37, 41].

### 3.3 Biological evaluation

#### 3.3.1 In vitro antitumor against HepG2 and Erlotinib-resistant HepG2 cell lines

All final NO-CIEAs 17, 20a-g, 23a,b, 25, 28 and 30 were tested for the anticancer activity against both HepG2 (liver cancer cells) and HepG2-R (Erlotinib resistant liver cancer cells) using MTT assay [42-44]. IC\(_{50}\) was calculated with regard to DMSO control group and potency was calculated with regard to percentage of antiproliferative activities of Erlotinib and tested compounds, as depicted, in Table 2.

As shown in Table 2, our SAR study showed that some of the tested compounds have high to moderate antitumor activity towards liver cell line (HepG2) with IC\(_{50}\) values ranges from 4.69-43.28 \( \mu \text{M} \) in comparison to Erlotinib as a reference drug (IC\(_{50} \) = 25 \( \mu \text{M} \)). The NO-CIEA 17 with a hydrophilic cucurbitacin side chain installed at C-17 position of the estrone ring, R configuration around C-19 and tethered with NO releasing furoxan moiety at C-3 position through an ether linkage exhibited the most potent anticancer activity (IC\(_{50} \) = 4.69 \( \mu \text{M} \)) among the NO-CIEAs as well as the reference drug Erlotinib. The data also revealed that 20a, (19R)-isomer, with strong electron withdrawing \( p \)-(trifluoromethyl)phenyl group revealed potent anticancer activity with IC\(_{50} \) 12.5 \( \mu \text{M} \) compared to reference drug Erlotinib (IC\(_{50} \) = 25 \( \mu \text{M} \)) while its diastereomeric S-isomer (compound 23b) was inactive up to 50 \( \mu \text{M} \).

Moreover, the (19S)-isomer 23a (IC\(_{50} \) = 43.82 \( \mu \text{M} \)) with electron donating \( p \)-(methoxy)phenyl group generally exhibited slightly better anticancer activity than the corresponding R-one (20a; (IC\(_{50} > 50 \mu \text{M} \)), demonstrating that the S configuration analog was a somewhat better substrate for cellular kinases than the R one in this series. Furthermore, introduction of a heterocyclic moiety, like substituted thiophenyl or furyl moieties, did not inhibit the proliferation of HepG2 cells at concentrations as high as 50 \( \mu \text{M} \), indicating that no
heterocyclic side chain at the C-17 position favored anticancer activity. Consequently, introducing an aliphatic hydrophilic cucurbitacin side chain at C-17 and tethering the nitric oxide releasing furoxan moiety at C-3 position may have an impact on the binding affinity towards the ATP binding site of EGFR and hydrogen bonding formation and thus increase the ability of the ligands to be more active.

The resistant hepatocellular cancer cells (HepG2-R) was developed in our research group against Erlotinib as standard drug [45]. Hence, all of the final NO-CIEAs were evaluated for their antitumor activity against HepG2-R to study the effect of target compounds on chemotherapeutic resistance using MTT assay. The data revealed that only one analog (17) still maintain its cytotoxicity activity against HepG2-R cell line with IC₅₀ 8.21 µM compared to sensitive HepG2 cell line (IC₅₀ = 4.69 µM).

For further investigation to study SAR of NO-CIEAs, the intermediates 25 and 28 having furoxan and organic dinitrate moieties, respectively, were tested against both HepG2 and HepG2-R cell lines. The data revealed that neither 25 nor 28 inhibited the proliferation at concentrations as high as 50 µM, indicating that either cucurbitacin or electron with drawing containing aromatic side chain at the C-17 position favored anticancer activity.

Based on variations in compounds’ activity related to their diversity in chemical features, the attention was moved to calculate some physiochemical preparties as CLogP and PSA [46, 47]. This increasing interest towards lipophilicity can be ascribed to its proven fundamental relevance in absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies. As a consequence, data on lipophilicity is currently being applied in structural information about the biological behavior of drug candidates [48]. As shown in Table 2. NO-CIEA 17 possesses good physicochemical properties (CLogP 6.57 and PSA 118.25) among the target final analogs with a potent broad-spectrum cytotoxicity activity against both HepG2 and HepG2-R cell lines with IC₅₀ values 4.69 µM and 8.21 µM, respectively. Also, higher, or lower CLogP or PSA values than that of NO-CIEA 17, showed the lowest cytotoxicity activity.

Our attention was then drawn to examine the coupled precursors of NO-CIEA 17 to determine their potential of cytotoxic activity. Hence, NO releasing furoxan alcohol 13 and compound 16 (des-NO analogs to compound 17) were examined (Table 3). The data revealed
that 13 did not inhibit the proliferation of both cell lines (IC$_{50} > 50$ µM) while 16 showed IC$_{50}$ 25 and 39.18 µM in HepG2 and HepG2-R cells, respectively, indicating that hybridization favored anticancer activity as well as blocking the estrogenic activity of phenolic derivative through methoxy linkage.

Therefore, tethering NO releasing moiety to cucurbitacin inspired estrone analogs (CIEAs) known of potent Anti-proliferation of HCC, will significantly impact the potential anticancer activity of hybrid drug candidate because of synergistic effects of both NO and CIEA. In this context, the designed synthesized novel NO-CIEAs are considered as promising candidates for treatment of HCC and overcoming the chemotherapeutic resistance.

3.3.2 Intracellular measurement of NO release

NO release behavior of compounds 17 and 20a was examined to indicate whether the strong inhibition of active compounds on the proliferation of tumor cells could be associated with high levels of NO production in HepG2 cells. The latter were exposed to each compound at different concentrations for varying durations (1, 6, 12 and 24 h). The levels of NO produced intracellularly from NO-CIEAs 17 and 20a along with JS-K (a known NO donor prodrug with two moles NO-releasing diazinium diolate moiety) were examined using NO-sensitive fluorophore, 4-amino-5-(methylamino)-2',7'-difluorofluorescein diacetate (DAF-FM DA) [43, 49-52], (Fig. 5A-C). It was observed that furoxan hybrids 17 and 20a showed an increase in fluorescence after 1 h of incubation in a dose-dependent manner and produced a significant concentration of NO in tumor cells comparable to JS-K (reference drug). Also, NO-CIEA 17 and 20a released maximum level of NO at a concentration of 32.92 and 25 µM in comparison to the reference prodrug JS-K which produced the maximum release at 50 µM. Furthermore, the data revealed that there is a significant decrease in NO level at 50 µM after incubation for 1 h with either NO-CIEA 17 or 20a. In addition, in a dose dependent manner, both NO-CIEAs 17 and 20a showed a marked decrease in NO level compared to DMSO control after incubation for 6, 12 and 24 h. Obviously, the incubation of cells with the NO-CIEAs 17 and 20a decreased the overall metabolic activity of the cells, as determined by MTT assay within the concentration range and incubation periods tested; thus, the low NO intracellular content at high concentration of NO donors was caused by their cytotoxicity activity [49]. Moreover, the DAF-FM DA probe was
sufficient for detecting the increased fluorescence within a wide range of NO concentrations as was proven by the measurements after 1 h of incubation. This finding document that any potential quenching of the specific DAF-FM DA fluorescence after incubation for long periods, by increased NO production at higher concentrations of NO-CIEAs 17 and 20a, did not happen.

Again, we found that among the NO-CIEAs tested, 17 was the most active cytotoxic agent (IC50 = 4.69 µM). These results suggest that compound 17 is a far superior source of intracellular NO in comparison with the reference prodrug JS-K.

Additionally, NO-CIEA 17 was tested for its NO releasing activity in HepG2-R cell line and showed the highest level of NO at IC50 (8.21 µM) after 1 h of incubation while higher concentrations showed a decrease in fluorescence again (Fig. 5D). Therefore, it is clear to demonstrate that the potent inhibitory effects of NO-CIEA 17 were related to the obvious release of NO in HCC cells line along with cucurbitacin steroidal moiety.

### 3.3.3 Cell cycle arrest

Flow cytometric analyses of NO-CIEAs 17 and 20a were conducted to study the effect of these compounds on cell cycle. HepG2 cells were incubated with NO-CIEAs 17 (4.69 µM), 20a (12.5 µM) and vehicle DMSO (0.01%) as control for 24 h and the cell cycle parameters were conducted. NO-CIEA 17 caused accumulation of cells in G0/G1 phase. As shown in Figure 6B, NO-CIEA 17 exhibited an increase in the fraction of cells in the G0/G1 phase (63.46% in comparison to 42.06% for the control). Also, it showed a decrease in the proportion of cells in the S phase (26.31% compared to 41.09%) and the G2/M phase (10.22% compared to 16.85%) (Fig. 6A, B). In case of NO-CIEA 20a, it exhibited also an increase in the fraction of cells in the G0/G1 phase (53.42% compared to 42.06% for the control) and a decrease in the proportion of cells in the S phase (32.00% compared to 41.09%) and the G2/M phase (14.58% compared to 16.85%) (Fig. 6A, C). Briefly, our data showed that both NO-CIEAs 17 and 20a arrested the cells in G0/G1 phase of cell cycle.

### 3.3.4 In-Cell Western analysis (In-Cell Based ELISA Assay)

The effect of the most cytotoxic analog NO-CIEA 17 on EGFR-MAPK signaling pathway was evaluated using In-Cell Western assay (ICW) which is also known as In-Cell Based
ELISA Assay (Fig. 7). ICW is an indirect ELISA assay offering a measurement for the levels of two proteins in whole fixed cells without the need for cell lysate. This assay offers a sensitive measurement to be used for adherent and non-adherent cells measuring protein phosphorylation and total protein levels by fluorometric detection method using Odyssey® scanner (LI-COR®) [53-57]. As shown in Fig. 7A-C, the Odyssey images show detection of total proteins regardless of phosphorylation status as well as detection of decreasing amounts of phospho-protein as a function of increasing NO-CIEA 17 concentrations while the graphical representations show the quantification of fluorescence. The data showed the potential activity for NO-CIEA 17 to inhibit the phosphorylation of EGFR, ERK1/2 and MEK1/2 in a dose-dependent manner by about 25%, 29% and 11% inhibition, respectively, compared to untreated cells (Control). Also, the data revealed that there is an initial activation of MEK1/2 phosphorylation at a dose of 1.17 µM and this might be attributed to a partial agonistic activity of NO-CIEA 17 at low doses, Fig. 7C. Thus, NO-CIEA 17 showed a significant dose dependent reduction of EGFR and ERK1/2 phosphorylation in treated cells. This data suggests that the binding ability of the NO-CIEA 17 to the ERK is correlated with the docking study and the cell signaling analysis. The higher cytotoxic activity over the cell lines as well as the proper binding towards ERK clearly support our hypothesis that compound 17 is a potential drug candidate for treatment of HCC. Therefore, the NO-CIEA 17 has a potential inhibitory activity towards the MAPK kinase pathway by inhibiting phosphorylated ERK.

3.3.5 Multidrug resistance protein (MRP2) inhibition in HepG2-R cell line

Liver cancer cells have the ability to develop resistance to structurally and mechanistically unrelated drugs over a period of time [58]. MRP2 is the second member of the MRP subfamily of ABC transporter and shares 49% amino acid identity with MRP1 but it has a different expression pattern. Also, MRP2 is highly expressed in the apical (canalicular) hepatocyte plasma membrane [29]. Recently, our research group has reported that MRP2 is overexpressed in HepG2-R cell line compared to sensitive one [31] and the expression level of MRP2 was increased in resistant HepG2 cell line to Erlotinib 93% comparing to β-Actin as loading control. It has been reported also by Morrow et al that MRP2 has been expressed in sensitive HepG2 cell line by western blot [59]. Herein, the MRP2 inhibition was evaluated using ICW [53-57]. In a consequence, our attention has been extended to study the effect of NO-CIEA
17 on the chemotherapeutic resistance through exploring its inhibitory effect for MRP2. As shown in Fig. 7D, compound 17 showed a significant reduction of MRP2 expression in a dose dependent manner with 26% inhibition compared to control. Therefore, the NO-CIEA 17 has a significant impact on the chemotherapeutic resistance along with potential inhibitory activity towards the MAPK kinase pathway.

4 Conclusion

In conclusion, a series of NO-CIEAs were designed, synthesized and evaluated for their anti-proliferative activity as well as their EGFR-ERK signaling pathway inhibitory activity against hepatocellular carcinoma. Among these compounds, NO-CIEA 17 showed a broad potent spectrum anti-proliferative activity against both HepG2 and HepG2-R cell lines (IC$_{50}$ = 4.69 and 8.21 µM, respectively) in comparison to the positive control Erlotinib (IC$_{50}$ = 25 and 47.16 µM, respectively). Also, NO-CIEA 20a with lipophilic electron-withdrawing group showed remarkably high inhibitory activity against HepG2 cell line (IC$_{50}$ = 12.5 µM) comparable to positive control Erlotinib (IC$_{50}$ = 25 µM) while NO-CIEA 23a with electron-donating group showed moderate activity (IC$_{50}$ = 43.28 µM). The results indicating that cucurbitacin side chain at C-17 position and NO releasing furoxan moiety linked through alkoxy bridge at C-3 position showed clear SAR. Intracellular measurements of NO using DAF-FM DA revealed that NO-CIEAs 17 and 20a produced significant amounts of NO after 1 h of incubation in tumor cells comparable to the reference prodrug JS-K. Flow cytometric analysis showed that NO-CIEAs 17 and 20a mainly arrested the HepG2 cells in the G0/G1 stage. In-Cell Based ELISA screening showed that NO-CIEA 17 has a potential inhibitory activity towards both EGFR and MAPK pathway by inhibiting both phosphorylated EGFR and ERK, respectively. This data suggests the binding ability of the NO-CIEA 17 to both EGFR and ERK to be well correlated along with the docking study and the cell signaling analysis. Also, it showed a significant reduction of MRP2 expression by about 26% in comparison to control. Therefore, the NO-CIEA 17 has a significant impact on the chemotherapeutic resistance along with potential inhibitory activity towards the EGFR-MAPK pathway.
5 Experimental procedures

5.1 Chemistry

\(^1\)H and \(^{13}\)C NMR spectra were acquired on a Bruker AVANCE-400 or 600 MHz NMR spectrometer, in DMSO-\(d_6\), CDCl\(_3\) or (CD\(_3\))\(_2\)CO using the solvent residual peak as the internal standard, with the reporting of coupling constants in Hz and the signal multiplicities are reported as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of triplets (dt), multiplet (m), or broad (br). HRMS data was obtained using EI ionization on a ThermoFinnigan MAT 95 XL mass spectrometer. TLC analysis was performed using pre-coated silica gel PE sheets. Products were purified via column chromatography using silica gel 40–63 um (230–400 mesh) and prep-plates (Altech preparative column, Econosil C18 10u, length 250 mm, ID 22 mm). All reagents and solvents were obtained from commercial suppliers and used as received. All chemical reactions requiring anhydrous conditions were performed with oven-dried glassware under an atmosphere of nitrogen.

Compounds 2-6 [37], 7a [37], 9-11 [61], 13 [38], 15 [37], 16 [37], 18a,c,d, 19a,c,d, 21a-c, 22a-c and 24 [37, 41] and 27 [37] were prepared as reported by our research group.

5.1.1 General procedure for preparation of the key intermediates (R/S) \(\alpha\)-hydroxy methyl ketones (7b)

To a stirred solution of cyanohydrin 6 (6.9468 g, 6.95 mmol) in a dry ether (20 mL), methyl lithium (McLi) (1.6 M in ether, 13.03 mL, 20.85 mmol) was added dropwise at 0 °C. The reaction mixture was allowed to stir for 2 h at 0 °C, then quenched by adding glacial acetic acid (2.6 mL) in one portion at 0 °C and allowed to stir for 30 min at 0 °C. Saturated sodium bicarbonate solution was added to neutralize the acidic mixture. Ethyl acetate was used to extract the aqueous layer (3x50 mL), dried under sodium sulfate anhydrous, then concentrated under \(\text{vacuo}\) [37]. The resulted diastereomers were purified by silica gel column chromatography (5% ethyl acetate in hexane) to give the key intermediates \(\alpha\)-hydroxyl ketone 7a (19R-isomer; 59 %) and 7b (19S-isomer; 4%).

**7b;** colorless crystals; yield 4%; \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) 6.89 (d, \(J = 8.4\) Hz, 1H), 6.42 (dd, \(J = 8.4, 2.6\) Hz, 1H), 6.36 (d, \(J = 2.6\) Hz, 1H), 3.49 (s, 1H), 2.66 – 2.56 (m, 2H), 2.14
(s, 3H), 2.02 – 1.86 (m, 3H), 1.76 – 1.65 (m, 3H), 1.60 – 1.55 (m, 1H), 1.49 (dt, J = 11.9, 3.2 Hz, 1H), 1.26 – 1.18 (m, 2H), 1.15 (s, 3H), 1.09 – 0.98 (m, 3H), 0.79 (s, 9H), 0.79 (s, 9H), 0.55 (s, 3H), 0.00 (s, 6H). 13C NMR (151 MHz, CDCl3) δ 212.88, 153.33, 137.81, 132.95, 125.98, 119.97, 117.13, 79.82, 56.41, 55.61, 43.73, 42.80, 38.73, 37.91, 29.63, 27.65, 26.41, 26.16, 25.73, 25.11, 22.86, 22.60, 18.19, 12.90, -4.37. 13C DEPT-NMR (151 MHz, CDCl3) δ 125.98, 119.97, 117.13, 56.41, 55.61, 43.73, 38.72 (inverted C), 37.91, 29.63 (inverted CH2), 27.65 (inverted CH2), 26.42, 26.16 (inverted CH2), 25.73, 25.11, 22.86 (inverted CH2), 22.60 (inverted CH2), 12.90, -4.37.

5.1.2 General procedure for preparation of 3-(((methylsulfonyl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (14)

Triethylamine (0.62 mL, 4.43 mmol) was added to furoxan alcohol (284 mg, 1.48 mmol) in dry CH2Cl2 (10 mL) at 0 °C followed by dropwise addition of MsCl (0.17 mL, 2.2 mmol) over 10 min. The mixture was allowed to warm to room temperature and stirred for 3 h. The reaction was quenched with saturated aqueous NH4OH (10 mL) and extracted with ethyl acetate (3×75 mL). Combined organic layers washed with brine (2×15 mL), dried over anhydrous Na2SO4, and concentrated under vacuo to give yellow oil [38]. The desired product was isolated by column chromatography (hexane/ethyl acetate; 9.5:0.5) as yellow oil which solidifies upon freezing yielding furoxan mesylate 14 as yellow crystals (53%). 1H NMR (400 MHz, CDCl3) δ 7.67 (m, 5H), 4.57 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 156.06, 131.66, 129.60, 127.56, 125.63, 113.07, 32.84.

5.1.3 General procedure for preparation of (R,E)-4-(((8S,9S,13S,14S,17S)-3-((tert-butyldimethylsilyloxy)-13,17-dimethyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-4-hydroxy-1-(aryl)pent-1-en-3-one (18b, 24a,b)

To a solution of α-hydroxy methyl ketone 7a,b (0.1836 g, 0.4 mmol) in THF (1 mL) in an open 20 mL-glass vial, substituted aromatic/heterocyclic aldehyde (0.8 mmol) and sodium hydroxide (0.0464 g, 1.16 mmol) were added at room temperature and the reaction mixture was then heated at 105 °C for 20-30 min with continuing addition of THF by an approximate rate of 0.5 mL/min (avoid dryness; TLC monitoring). The reaction mixture was then quenched by the addition of water (15 mL), followed by extraction with EtOAc (3x20 mL), washed with brine, dried with anhydrous Na2SO4, and concentrated in vacuo [37, 62-64]. The crude product was
purified by silica gel column chromatography with hexanes/EtOAc (9.5:0.5) to yield the corresponding TBS protected enones 18b and 24a,b.

5.1.3.1 (R,E)-4-((8S,9S,13S,14S,17S)-3-((tert-butyldimethylsilyl)oxy)-13,17-dimethyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-4-hydroxy-1-(4-(methylthio)phenyl)pent-1-en-3-one (18b)

White solid; 43% yield; 1H NMR (600 MHz, CDCl3) δ 7.61 (d, J = 15.5 Hz, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 1H), 6.43 (dd, J = 8.4, 2.5 Hz, 1H), 6.36 (d, J = 2.5 Hz, 1H), 4.03 (s, 1H), 2.66 – 2.56 (m, 2H), 2.31 (s, 3H), 2.16 – 1.99 (m, 3H), 1.71 – 1.62 (m, 2H), 1.47 – 1.38 (m, 3H), 1.36 (s, 3H), 1.35 – 1.32 (m, 1H), 1.15 – 1.03 (m, 5H), 0.79 (s, 9H), 0.78 (s, 3H), -0.00 (s, 6H). 13C NMR (151 MHz, CDCl3) δ 201.87, 153.34, 145.18, 143.12, 137.87, 133.17, 130.74, 129.05, 126.05, 125.91, 120.01, 117.31, 117.17, 79.15, 55.79, 55.17, 44.36, 43.92, 40.76, 38.11, 29.66, 27.68, 26.64, 25.78, 24.43, 23.70, 22.08, 18.23, 15.10, 13.70, -4.32.

5.1.3.2 (S,E)-4-((8S,9S,13S,14S,17S)-3-((tert-butyldimethylsilyl)oxy)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-4-hydroxy-1-(4-methoxyphenyl)pent-1-en-3-one (21a)

White solid; 15% yield; 1H NMR (400 MHz, CDCl3) δ 7.67 (d, J = 15.6 Hz, 1H), 7.43 (d, J = 8.7 Hz, 2H), 6.86 – 6.75 (m, 4H), 6.42 – 6.33 (m, 2H), 3.81 (s, 1H), 3.68 (s, 3H), 2.65 – 2.62 (m, 2H), 2.05 – 1.74 (m, 3H), 1.73 – 1.55 (m, 2H), 1.52 – 1.33 (m, 3H), 1.22 (s, 3H), 1.19 – 1.02 (m, 6H), 0.80 (s, 9H), 0.61 (s, 3H), 0.04 – 0.00 (m, 6H). 13C NMR (101 MHz, CDCl3) δ 202.78, 162.01, 153.26, 144.14, 137.79, 133.10, 130.47, 127.18, 126.03, 119.92, 117.76, 117.10, 114.54, 79.07, 56.38, 55.67, 55.47, 43.76, 43.06, 39.25, 37.95, 29.69, 27.70, 26.54, 25.74, 22.93, 22.69, 18.18, 14.18, 13.37, -4.37.

5.1.3.3 (S,E)-4-((8S,9S,13S,14S,17S)-3-((tert-butyldimethylsilyl)oxy)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-4-hydroxy-1-(4-(trifluoromethyl)phenyl)pent-1-en-3-one (24b)

White solid; 38% yield; 1H NMR (600 MHz, CDCl3) δ 7.43 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 8.2 Hz, 2H), 6.86 (d, J = 8.5 Hz, 1H), 6.68 (d, J = 15.8 Hz, 1H), 6.41 (dd, J = 8.5, 2.5 Hz, 2H), 6.36 (d, J = 2.5 Hz, 1H), 4.04 – 3.94 (m, 2H), 2.54 – 2.46 (m, 2H), 2.30 – 2.12 (m, 2H), 1.73 – 1.64 (m, 2H), 1.19 – 1.10 (m, 5H), 0.79 (s, 9H), 0.77 (s, 3H), -0.00 (s, 6H). 13C NMR (151 MHz, CDCl3) δ 201.87, 153.34, 145.18, 143.12, 137.87, 133.17, 130.74, 129.05, 126.05, 125.91, 120.01, 117.31, 117.17, 79.15, 55.79, 55.17, 44.36, 43.92, 40.76, 38.11, 29.66, 27.68, 26.64, 25.78, 24.43, 23.70, 22.08, 18.23, 15.10, 13.70, -4.32.
1H), 6.37 (d, J = 2.5 Hz, 1H), 6.23 (d, J = 15.8 Hz, 1H), 4.25 (s, 1H), 2.69 – 2.59 (m, 2H), 2.10 – 1.93 (m, 2H), 1.85 – 1.80 (m, 2H), 1.73 – 1.68 (m, 2H), 1.54 – 1.50 (m, 1H), 1.36 (s, 3H), 1.34 – 1.28 (m, 2H), 1.24 – 1.09 (m, 5H), 0.79 (s, 9H), 0.66 (s, 3H), -0.00 (s, 6H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 213.04, 153.42, 139.74, 137.62, 132.56, 131.70, 129.82, 126.76, 126.07, 125.73, 125.71, 119.98, 117.22, 79.70, 56.23, 55.91, 47.42, 43.58, 39.76, 38.59, 29.58, 28.96, 27.91, 26.53, 25.73, 25.10, 24.87, 18.20, 13.82, -4.37.

5.1.4 General procedure for preparing of (R,E)-4-hydroxy-4-((8S,9S,13S,14S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1-(aryl or Het.)pent-1-en-3-one (19b and 22a,b)

To a stirred solution of protected enones 7a, 16 and 21a,b (0.18 mmol) in THF (5 ml), TBAF (0.53 ml, 0.53 mmol, 1 M in THF) was added and stirred for 12 h. The reaction mixture was then quenched with saturated ammonium chloride solution and extracted with ethyl acetate (3x20 mL), washed with brine, dried over anhydrous sodium sulfate, filtrated, and concentrated under vacuo [37]. Silica gel column chromatography was used to purify the crude material (hexanes/EtOAc; 8:2) to provide the corresponding phenolic enones 19b and 22a,b.

5.1.4.1 (R,E)-4-hydroxy-4-((8S,9S,13S,14S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1-(4-(methylthio)phenyl)pent-1-en-3-one (19b)

White solid; 50% yield; $^1$H NMR (600 MHz, CDCl$_3$) δ 7.72 (d, J = 15.6 Hz, 1H), 7.44 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.5 Hz, 2H), 7.07 (d, J = 8.4 Hz, 1H), 6.92 (d, J = 15.6 Hz, 1H), 6.56 (dd, J = 8.4, 2.7 Hz, 1H), 6.48 (d, J = 2.7 Hz, 1H), 5.16 (s, 1H), 4.22 (s, 1H), 2.77 – 2.67 (m, 2H), 2.43 (s, 3H), 2.27 – 2.09 (m, 3H), 1.82 – 1.73 (m, 2H), 1.57 – 1.50 (m, 3H), 1.49 (s, 3H, C20-H3), 1.35 – 1.32 (m, 1H), 1.24 – 1.15 (m, 5H), 0.89 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 201.95, 153.47, 145.40, 143.19, 138.26, 132.64, 130.67, 129.08, 126.44, 125.89, 117.19, 115.32, 112.70, 79.27, 55.71, 55.17, 44.33, 43.84, 40.69, 38.13, 29.64, 27.58, 26.67, 24.34, 23.65, 22.05, 15.09, 13.67.
5.1.4.2 (S,E)-4-hydroxy-4-((8S,9S,13S,14S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1-(4-methoxyphenyl)pent-1-en-3-one (22a)

White solid; 65% yield; $^1$H NMR (400 MHz, Acetone) $\delta$ 7.75 (s, 1H), 7.60 – 7.56 (m, 3H, Ar-H), 7.19 (d, $J = 15.7$ Hz, 1H), 6.82 (d, $J = 8.9$ Hz, 2H), 6.79 (d, $J = 8.4$ Hz, 1H), 6.35 (dd, $J = 8.4$, 2.6 Hz, 1H), 6.31 (d, $J = 2.6$ Hz, 1H), 3.82 (s, 1H), 3.67 (s, 3H), 2.64 – 2.47 (m, 2H), 2.02 – 1.89 (m, 2H), 1.78 – 1.61 (m, 2H), 1.60 – 1.26 (m, 3H), 1.16 (s, 3H), 1.13 – 0.96 (m, 6H), 0.58 (s, 1H). $^{13}$C NMR (101 MHz, Acetone) $\delta$ 162.93, 155.92, 144.05, 138.33, 131.99, 131.51, 128.38, 126.99, 119.52, 115.92, 115.33, 113.55, 79.82, 56.64, 56.28, 55.86, 44.60, 43.68, 39.96, 39.17, 28.52, 27.17, 26.98, 23.63, 23.16, 13.95.

5.1.4.3 (S,E)-4-hydroxy-4-((8S,9S,13S,14S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1-(4-(trifluoromethyl)phenyl)pent-1-en-3-one (22b)

White solid; 63% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.53 (d, $J = 8.2$ Hz, 2H), 7.42 (d, $J = 8.2$ Hz, 2H), 6.98 (d, $J = 8.4$ Hz, 1H), 6.77 (d, $J = 15.9$ Hz, 1H), 6.51 (dd, $J = 8.4$, 2.6 Hz, 1H), 6.47 (d, $J = 2.6$ Hz, 1H), 6.32 (d, $J = 15.9$ Hz, 1H), 4.83 (s, 1H), 4.40 (s, 1H), 2.80 – 2.66 (m, 2H), 2.09 – 1.90 (m, 3H), 1.83 – 1.78 (m, 2H), 1.63 – 1.61 (m, 1H), 1.46 (s, 3H), 1.43 – 1.10 (m, 8H), 0.76 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 213.05, 153.45, 138.05, 132.15, 131.58, 129.92, 126.86, 126.77, 126.47, 125.73, 125.71, 115.27, 112.72, 79.75, 56.21, 55.83, 47.40, 43.49, 39.70, 38.62, 29.56, 28.96, 27.80, 26.60, 25.03, 24.85, 13.79.

5.1.5 General procedure for preparing of 3-(((8S,9S,13S,14S,17S)-17-((R,E)-5-(aryl or Het.)-2-hydroxy-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (17, 20a-g, 23a,b and 25)

To a stirred solution of phenolic enones 16, 19a-g, 22a,b and 24 (0.28 mmol) in THF (10 mL), sodium hydroxide (0.011 g, 0.28 mmol) and furoxan mesylate 14 (0.150 g, 0.56 mmol) were added. The reaction mixture was heated under reflux for 1 h. then another 0.011 g (0.28 mmol) of sodium hydroxide was added and the reflux was continued for another 1 h (TLC
monitoring). The reaction mixture was quenched with water and extracted with ethyl acetate (3x20 mL), washed with brine, dried over sodium sulfate anhydrous, filtrated, and concentrated under vacuo [38, 65-67]. Silica gel column chromatography was used to purify the crude materials to afford the corresponding titled NO-CIEAs (30% ethyl acetate in hexane for 17 and 15% ethyl acetate in hexane for 20a-g, 23a,b and 25).

5.1.5.1 3-(((8S,9S,13S,14S)-17-((R,E)-2,6-dihydroxy-6-methyl-3-oxohept-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (17)

Yellowish white solid; 34% yield; ¹H NMR (600 MHz, CDCl₃) δ 7.79 (dd, J = 8.2, 1.4 Hz, 2H), 7.49 – 7.42 (m, 3H), 7.15 (d, J = 8.5 Hz, 1H), 7.08 (d, J = 15.2 Hz, 1H), 6.71 (dd, J = 8.5, 2.8 Hz, 1H), 6.64 (d, J = 2.8 Hz, 1H), 6.62 (d, J = 15.2 Hz, 1H), 4.99 (s, 2H), 4.05 (s, 1H), 2.82 – 2.72 (m, 2H), 2.27 – 2.19 (m, 2H), 2.16 – 2.10 (m, 1H), 1.97 (s, 1H), 1.81 – 1.74 (m, 2H), 1.62 – 1.56 (m, 2H), 1.52 – 1.48 (m, 3H), 1.42 (s, 3H), 1.33 (s, 6H), 1.18 (m, 3H), 0.87 (s, 3H), 0.80 (dd, J = 7.0, 3.2 Hz, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 202.34, 157.16, 156.02, 154.84, 138.55, 134.60, 131.36, 129.35, 127.78, 126.65, 126.26, 118.10, 114.93, 112.28, 112.25, 79.19, 71.36, 58.32, 55.63, 54.62, 44.28, 43.83, 40.55, 37.99, 29.76, 29.56, 29.51, 27.49, 26.59, 24.17, 23.67, 21.98, 13.63. HRESI-MS m/z calcd for [M+Na]+ C₃₅H₄₂N₂O₆: 609.293508, found: 609.295609.

5.1.5.2 3-(((8S,9S,13S,14S,17S)-17-((R,E)-2-hydroxy-3-oxo-5-(4-(trifluoromethyl)phenyl)pent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20a)

Yellow solid; 69% yield; ¹H NMR (600 MHz, CDCl₃) δ 7.81 – 7.79 (m, 2H), 7.77 (d, J = 15.7 Hz, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.47 (m, 3H), 7.17 (d, J = 8.6 Hz, 1H), 7.04 (d, J = 15.7 Hz, 1H), 6.73 (dd, J = 8.6, 2.8 Hz, 1H), 6.65 (d, J = 2.8 Hz, 1H), 5.00 (s, 2H), 4.00 (s, 1H), 2.79 – 2.74 (m, 2H), 2.30 – 2.14 (m, 3H), 1.82 – 1.78 (m, 2H), 1.58 – 1.53 (m, 3H), 1.50 (s, 3H), 1.40 – 1.35 (m, 1H), 1.23 – 1.17 (m, 5H), 0.90 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 201.72, 157.15, 154.88, 143.64, 138.52, 137.73, 134.51, 131.36, 129.34, 128.77, 127.75, 126.63, 126.26, 125.95, 125.92, 120.84, 114.94, 112.27, 112.22, 79.39, 58.30, 55.68,
55.03, 44.35, 43.83, 40.62, 38.00, 31.97, 29.75, 29.71, 29.41, 22.73, 14.16, 13.65. HRESI-MS m/z calcd for [M+H]^+ C_{39}H_{39}F_3N_2O_5: 673.288400, found: 673.287550.

**5.1.5.3** 3-(((8S,9S,13S,14S,17S)-17-((R,E)-2-hydroxy-5-(4-(methylthio)phenyl)-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20b)

Yellowish white solid; 62% yield; ^1H NMR (600 MHz, CDCl₃) δ 7.81 – 7.76 (m, 2H), 7.50 – 7.41 (m, 5H), 7.19 – 7.12 (m, 3H), 6.91 (d, J = 15.5 Hz, 1H), 6.72 (dd, J = 8.6, 2.8 Hz, 1H), 6.63 (d, J = 2.7 Hz, 1H), 4.98 (s, 2H), 4.14 (s, 1H), 2.84 – 2.67 (m, 2H), 2.43 (s, 3H), 2.31 – 2.10 (m, 3H), 1.84 – 1.74 (m, 2H), 1.62 – 1.49 (m, 3H), 1.47 (s, 3H), 1.39 – 1.33 (m, 1H), 1.30 – 1.12 (m, 5H), 0.89 (s, 3H). ^13C NMR (151 MHz, CDCl₃) δ 201.84, 157.16, 154.85, 145.21, 143.14, 138.59, 134.62, 131.37, 130.71, 129.36, 129.04, 127.78, 126.65, 126.27, 125.90, 117.26, 114.95, 112.28, 112.24, 79.11, 58.33, 55.70, 55.15, 44.30, 43.86, 40.65, 38.01, 29.77, 27.50, 26.62, 24.41, 23.66, 22.04, 15.09, 13.64. HRESI-MS m/z calcd for [M+Na]^+ C_{38}H_{39}FN_2O_5: 673.270664, found: 673.273272.

**5.1.5.4** 3-(((8S,9S,13S,14S,17S)-17-((R,E)-5-(4-fluorophenyl)-2-hydroxy-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20c)

Yellowish white solid; 73% yield; ^1H NMR (600 MHz, CDCl₃) δ 7.78 – 7.75 (m, 2H), 7.50 – 7.41 (m, 2H), 7.47 – 7.39 (m, 3H), 7.14 (d, J = 15.5 Hz, 1H), 7.04 – 6.99 (m, 2H), 6.90 (d, J = 15.6 Hz, 1H), 6.70 (dd, J = 8.6, 2.8 Hz, 1H), 6.62 (d, J = 2.8 Hz, 1H), 4.96 (s, 2H), 4.08 (s, 1H), 2.79 – 2.69 (m, 2H), 2.28 – 2.09 (m, 3H), 1.83 – 1.73 (m, 2H), 1.58 – 1.50 (m, 3H), 1.47 (s, 3H), 1.37 – 1.31 (m, 1H), 1.26 – 1.14 (m, 5H), 0.88 (s, 3H). ^13C NMR (151 MHz, CDCl₃) δ 201.79, 157.17, 154.88, 144.41, 138.57, 134.59, 131.38, 130.71, 130.65, 129.37, 127.78, 126.66, 126.27, 118.20, 116.31, 116.16, 114.96, 112.29, 112.25, 79.19, 58.34, 55.71, 55.10, 44.32, 43.86, 40.65, 38.01, 29.76, 27.51, 26.62, 24.37, 23.67, 22.07, 13.66. HRESI-MS m/z calcd for [M+Na]^+ C_{38}H_{39}FN_2O_5: 645.273521, found: 645.275728.
5.1.5.5 3-(((8S,9S,13S,14S,17S)-17-((R,E)-2-hydroxy-5-(4-methoxyphenyl)-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20d)

White solid; 59% yield; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.89 (dd, $J = 7.9$, 1.4 Hz, 2H), 7.85 (d, $J = 15.5$ Hz, 1H), 7.63 – 7.54 (m, 5H), 7.27 (d, $J = 15.5$ Hz, 1H), 6.97 (d, $J = 1.5$ Hz, 2H), 6.94 (d, $J = 8.5$ Hz, 1H), 6.83 (dd, $J = 8.5$, 2.5 Hz, 1H), 6.74 (d, $J = 2.5$ Hz, 1H), 6.09 (s, 2H), 4.31 (s, 1H), 3.88 (s, 3H), 2.86 (q, $J = 10.6$ Hz, 2H), 2.36 (dd, $J = 20.6$, 7.9 Hz, 2H), 2.28 – 2.21 (m, 1H), 1.90 (dt, $J = 8.5$, 6.2 Hz, 2H), 1.69 – 1.63 (m, 3H), 1.58 (s, 3H), 1.47 (d, $J = 10.2$ Hz, 1H), 1.33 (dd, $J = 17.1$, 10.0 Hz, 5H), 1.00 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 201.84, 162.04, 157.16, 154.84, 145.58, 138.59, 134.63, 131.37, 130.54, 129.36, 127.77, 127.01, 126.65, 126.25, 115.94, 114.93, 114.47, 112.24, 79.00, 58.30, 55.69, 55.48, 55.18, 44.26, 43.86, 40.65, 38.00, 29.76, 27.50, 26.62, 24.45, 23.66, 22.02, 13.62. HRESI-MS m/z calcd for [M+H]$^+$ C$_{39}$H$_{42}$N$_2$O$_6$: 635.311600, found: 635.310370.

5.1.5.6 3-(((8S,9S,13S,14S,17S)-17-((R,E)-2-hydroxy-5-(5-methylthiophen-2-yl)-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20e)

Yellow solid; 45% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.81 – 7.77 (m, 3H), 7.48 – 7.42 (m, 3H), 7.16 (d, $J = 8.6$ Hz, 1H), 7.09 (d, $J = 3.6$ Hz, 1H), 6.72 (dd, $J = 8.6$, 2.7 Hz, 1H), 6.68 (dd, $J = 3.6$, 1.0 Hz, 1H), 6.63 (d, $J = 2.7$ Hz, 1H), 6.58 (d, $J = 15.2$ Hz, 1H), 4.98 (s, 2H), 4.15 (s, 1H), 2.79 – 2.71 (m, 2H), 2.45 (s, 3H), 2.27 – 2.12 (m, 3H), 1.80 – 1.74 (m, 2H), 1.60 – 1.40 (m, 3H), 1.38 – 1.32 (m, 1H), 1.30 – 1.12 (m, 5H), 0.88 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 201.58, 157.16, 154.85, 145.34, 138.60, 138.49, 137.82, 134.66, 133.69, 131.37, 129.36, 127.97, 127.03, 126.66, 126.27, 115.87, 114.94, 112.27, 112.24, 78.94, 58.32, 55.66, 55.20, 44.26, 43.84, 40.62, 38.01, 29.78, 27.50, 26.62, 24.44, 23.66, 22.03, 15.99, 13.61. HRESI-MS m/z calcd for [M+Na]$^+$ C$_{37}$H$_{40}$N$_2$O$_5$S: 647.255014, found: 647.256570.
5.1.5.7 3-(((8S,9S,13S,14S,17S)-17-((R,E)-5-(5-bromothiophen-2-yl)-2-hydroxy-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20f)

Yellowish white solid; 72% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.80 – 7.77 (m, 2H), 7.73 (d, $J = 15.2$ Hz, 1H), 7.49 – 7.42 (m, 3H), 7.16 (d, $J = 8.6$ Hz, 1H), 7.02 (d, $J = 3.9$ Hz, 1H), 6.98 (d, $J = 3.9$ Hz, 1H), 6.72 (dd, $J = 8.6$, 2.8 Hz, 1H), 6.64 – 6.61 (m, 2H), 4.99 (s, 2H), 4.03 (s, 1H), 2.81 – 2.71 (m, 2H), 2.27 – 2.11 (m, 3H), 1.81 – 1.72 (m, 2H), 1.60 – 1.51 (m, 3H), 1.44 (s, 3H), 1.38 – 1.33 (m, 1H), 1.29 – 1.12 (m, 5H), 0.88 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 201.43, 157.16, 154.85, 141.33, 138.57, 137.05, 134.60, 133.00, 131.37, 129.36, 127.78, 126.66, 126.27, 117.54, 117.12, 114.94, 112.28, 112.24, 79.12, 58.32, 55.64, 55.11, 44.30, 43.82, 40.60, 38.01, 29.77, 27.49, 26.61, 24.34, 23.65, 22.05, 13.62. HRESI-MS m/z calcd for [M+Na]$^+$ C$_{36}$H$_{37}$BrN$_2$O$_5$S: 711.149876, found: 711.153027.

5.1.5.8 3-(((8S,9S,13S,14S,17S)-17-((R,E)-5-(5-bromofuran-2-yl)-2-hydroxy-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (20g)

Brown solid; 65% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.78 – 7.74 (m, 2H), 7.46 – 7.40 (m, 3H), 7.36 (d, $J = 15.2$ Hz, 1H), 7.14 (d, $J = 8.6$ Hz, 1H), 6.80 (d, $J = 15.2$ Hz, 1H), 6.70 (dd, $J = 8.6$, 2.6 Hz, 1H), 6.62 (d, $J = 2.6$ Hz, 1H), 6.57 (d, $J = 3.5$ Hz, 1H), 6.36 (d, $J = 3.5$ Hz, 1H), 4.96 (s, 2H), 4.07 (s, 1H), 2.78 – 2.69 (m, 2H), 2.25 – 2.11 (m, 3H), 1.81 – 1.73 (m, 2H), 1.57 – 1.47 (m, 3H), 1.44 (s, 3H), 1.34 – 1.30 (m, 1H), 1.27 – 1.11 (m, 5H), 0.86 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 201.62, 157.16, 154.87, 141.33, 138.57, 137.05, 134.64, 133.00, 131.37, 129.36, 127.78, 126.68, 126.42, 126.28, 119.17, 116.39, 114.94, 114.84, 112.29, 112.25, 79.12, 58.33, 55.64, 55.11, 44.30, 43.82, 40.60, 38.01, 29.77, 27.49, 26.61, 24.34, 23.65, 22.05, 13.62. HRESI-MS m/z calcd for [M+Na]$^+$ C$_{36}$H$_{37}$BrN$_2$O$_5$: 695.172720, found: 695.175669.
5.1.5.9 3-(((8S,9S,13S,14S,17S)-17-((S,E)-2-hydroxy-5-(4-methoxyphenyl)-3-oxopent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (23a)

Yellow solid; 39% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.79 – 7.76 (m, 2H), 7.52 (d, $J = 8.7$ Hz, 2H), 7.48 – 7.41 (m, 3H), 7.04 (d, $J = 8.6$ Hz, 1H), 6.89 – 6.85 (m, 3H), 6.66 (dd, $J = 8.6$, 2.5 Hz, 1H), 6.62 (d, $J = 2.5$ Hz, 1H), 4.96 (s, 2H), 3.89 (s, 1H), 3.79 (s, 3H), 2.79 – 2.72 (m, 2H), 2.10 – 1.98 (m, 3H), 1.90 – 1.80 (m, 2H), 1.72 – 1.48 (m, 3H), 1.31 (s, 3H), 1.27 – 1.13 (m, 6H), 0.70 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 202.76, 162.02, 157.15, 154.80, 144.19, 138.53, 134.57, 131.34, 130.47, 129.34, 127.76, 127.16, 126.61, 126.25, 117.71, 114.87, 114.54, 112.24, 112.21, 79.05, 58.28, 56.35, 55.59, 55.50, 43.70, 43.02, 39.16, 37.85, 29.79, 27.51, 26.51, 26.27, 22.89, 22.66, 13.32. HRESI-MS m/z calcd for [M+H]$^+$ C$_{39}$H$_{42}$N$_2$O$_6$: 635.311564, found: 635.313847.

5.1.5.10 3-(((8S,9S,13S,14S,17S)-17-((S,E)-2-hydroxy-3-oxo-5-(4-(trifluoromethyl)phenyl)pent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (23b)

Yellow solid; 34% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.78 (m, 3H), 7.53 (d, $J = 8.1$ Hz, 2H), 7.50 – 7.39 (m, 8H), 7.07 (d, $J = 8.6$ Hz, 1H), 6.77 (d, $J = 15.8$ Hz, 1H), 6.68 (dd, $J = 8.6$, 2.4 Hz, 1H), 6.64 (d, $J = 2.4$ Hz, 1H), 6.33 (d, $J = 15.8$ Hz, 1H), 4.97 (s, 2H), 4.34 (s, 1H), 2.79 – 2.74 (m, 2H), 2.11 – 2.03 (m, 2H), 1.95 – 1.90 (m, 2H), 1.84 – 1.78 (m, 2H), 1.64 – 1.62 (d, 1H), 1.52 – 1.50 (m, 1H), 1.46 (s, 3H), 1.42 – 1.22 (m, 6H), 0.76 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 212.98, 157.14, 154.91, 138.34, 134.02, 131.66, 131.37, 129.86, 129.35, 127.76, 126.85, 126.76, 126.69, 126.24, 125.74, 125.72, 114.90, 112.36, 112.20, 79.70, 58.29, 56.18, 55.80, 47.32, 43.52, 39.64, 38.50, 29.70, 28.93, 27.74, 26.54, 25.08, 24.84, 13.78. HRESI-MS m/z calcd for [M+Na]$^+$ C$_{39}$H$_{39}$F$_3$N$_2$O$_5$: 695.270328, found: 695.272678.
5.1.5.11 3-(((8S,9S,13S,14S)-17-((R)-2-hydroxy-3-oxobutan-2-yl)-13-methyl-
7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)methyl)-
4-phenyl-1,2,5-oxadiazole 2-oxide (25)

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.85 (dd, $J = 8.0$, 1.6 Hz, 2H), 7.56 – 7.48 (m, 3H), 7.22
(d, $J = 8.6$ Hz, 1H), 6.78 (dd, $J = 8.6$, 2.7 Hz, 1H), 6.71 (d, $J = 2.7$ Hz, 1H), 5.05 (s, 2H), 3.99 (s, 1H), 2.88 – 2.80 (m, 2H), 2.32 – 2.20 (m, 6H), 1.89 – 1.79 (m, 2H), 1.57 (m, 3H), 1.47 (s, 3H), 1.34 – 1.23 (m, 6H), 0.92 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 211.83, 157.16, 154.88, 138.53, 134.54, 131.39, 129.37, 127.76, 126.65, 126.25, 114.94, 112.28, 80.14, 58.32, 55.67, 55.10, 44.20, 43.80, 40.59, 37.96, 29.77, 27.52, 26.59, 24.63, 23.73, 23.35, 22.11, 13.53. $^{13}$C DEPT-NMR (101 MHz, CDCl$_3$) δ 131.45, 129.36, 127.76, 126.64, 114.93, 112.28, 58.31 (inverted), 55.72, 55.15, 43.80 (inverted), 40.66, 37.97, 37.95 (inverted), 29.76 (inverted), 27.50, 26.57 (inverted), 24.62, 23.70 (inverted), 23.33, 22.09 (inverted).

5.1.6 General procedure for preparing of (R)-3-((8S,9S,13S,14S,17S)-3-(allyloxy)-13-methyl-
7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-3-
hydroxybutan-2-one (26)

To a stirred solution of phenolic derivative 24 (0.2066 g, 0.37 mmol) in THF (2 ml), allyl bromide (0.0895, 0.74 mmol) and NaOH (0.0296 g, 0.74 mmol) were added. The reaction mixture was heated under reflux at 80 °C for 2 h (as indicated by TLC). After completion, water (15 ml) was added and the reaction mixture was extracted with ethyl acetate (3x10 mL), washed with brine, dried over anhydrous sodium sulphate, filtered and evaporated under vaccu (80 °C) to afford 3-allyloxy-estrone derivative 26 [65-67].

White solid; 85% yield; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.11 (d, $J = 8.6$ Hz, 1H), 6.65 (dd, $J = 8.6$, 2.5 Hz, 1H), 6.57 (d, $J = 2.5$ Hz, 1H), 5.98 (ddt, $J = 17.2$, 10.5, 5.3 Hz, 1H), 5.33 (dd, $J = 17.2$, 1.6 Hz, 1H), 5.19 (dd, $J = 10.5$, 1.6 Hz, 1H), 4.50 – 4.36 (m, 2H), 3.91 (s, 1H), 2.88 – 2.69 (m, 2H), 2.24 – 2.19 (m, 2H), 2.16 (s, 3H), 1.82 – 1.71 (m, 2H), 1.64 – 1.47 (m, 3H), 1.40 (s, 3H), 1.35 – 1.16 (m, 7H), 0.85 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 211.80, 156.49, 137.95, 133.60, 132.83, 126.20, 117.46, 114.71, 112.22, 80.13, 68.80, 55.72, 55.11, 44.24, 43.82, 40.67, 38.06, 29.81, 27.63, 26.59, 24.61, 23.70, 23.32 (COCH$_3$), 22.09, 13.51.
5.1.7 General procedure for preparing of (3R)-3-((8S,9S,13S,14S,17S)-3-(2,3-diiodopropoxy)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-3-hydroxybutan-2-one (27)

Iodine (0.95 g, 0.373 mmol) was added in one portion to a stirred solution of 3-allyloxy derivative 26 (0.1427 g, 0.373 mmol) and AgNO₃ (0.190 g, 1.120 mmol) in CH₃CN (2.5 ml). The reaction mixture was stirred at room temperature for 30 min., then filtered, diluted with distilled water (7.5 ml), and extracted with EtOAc (3x10 mL). The combined organic layers were washed with distilled water, brine, dried over anhydrous Na₂SO₄ and evaporated under vacuo. The resulting yellow oil was purified by flash chromatography (Hexane/EtOAc, 8:2) to yield the title product as a yellow oily diastereomeric mixture in the ratio about 1:1 (NMR detection) [67, 68].

Yellow oil; 86% yield; ¹H NMR (600 MHz, CDCl₃) δ 7.13 (d, J = 8.5 Hz, 1H), 6.63 (dd, J = 8.5, 2.6 Hz, 1H), 6.55 (d, J = 2.6 Hz, 1H), 4.77 (dd, J = 6.4, 2.6 Hz, 1H), 4.23 – 4.18, 4.17 – 4.09 (two m, 2H), 3.91 (s, 1H), 3.45, 3.37 (two dd, J = 10.8, 6.4, 10.8, 5.3 Hz, 2H), 2.79 – 2.71 (m, 2H), 2.24 – 2.18 (m, 2H), 2.16 (s, 3H), 1.82 – 1.72 (m, 2H), 1.65 – 1.59 (m, 1H), 1.51 – 1.46 (m, 3H), 1.40 (s, 3H), 1.36 – 1.14 (m, 6H), 0.85 (s, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 213.08, 156.96, 156.79, 139.65, 139.63, 135.29, 127.78, 127.77, 116.11, 116.00, 113.40, 113.37, 81.42, 81.26, 74.87, 67.96, 56.98, 56.40, 45.51, 45.09, 41.91, 39.29, 31.07, 28.84, 27.89, 25.91, 25.00, 24.63, 23.39, 20.94, 14.81.

5.1.8 General procedure for preparing of 3-(((8S,9S,13S,14S,17S)-17-((R)-2-hydroxy-3-oxobutan-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)propane-1,2-diyl dinitrate (28)

To a stirred solution of diiodo derivative 27 (0.2374 g, 0.373 mmol) in CH₃CN (2.5 ml), AgNO₃ (0.190 g, 1.120 mmol) in CH₃CN (2.5 ml) was added. The reaction mixture was stirred at room temperature for 1 h, then refluxed for 17 h (as indicated by TLC). After cooling, the mixture was filtered, diluted with distilled water (7.5 ml), and extracted with EtOAc (3x10 mL). The combined organic layers were washed with distilled water, brine, dried and evaporated. The resulting yellow oil was partly purified by flash chromatography (Hexane/EtOAc, 8:2) to yield
the title product as a yellow oily diastereomeric mixture in the ratio about 1:0.5 (NMR detection) [67, 68].

Yellow oil; 37% yield; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.15 (d, \(J = 8.7 \text{ Hz}, 1\text{H}\)), 6.62 (dd, \(J = 8.7, 2.5 \text{ Hz}, 1\text{H}\)), 6.55 (d, \(J = 2.5 \text{ Hz}, 1\text{H}\)), 5.62 – 5.47 (m, 1H), 4.85, 4.70 (two dd, \(J = 12.9, 3.3, 12.9, 6.6 \text{ Hz}, 2\text{H}\)), 4.21 – 4.09 (m, 2H), 3.92 (s, 1H), 2.80 – 2.73 (m, 2H), 2.27 – 2.18 (m, 3H), 2.16 (s, 3H), 1.83 – 1.73 (m, 2H), 1.65 – 1.47 (m, 3H), 1.40 (s, 3H), 1.35 – 1.14 (m, 6H), 0.86 (s, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 211.76, 211.71, 155.44, 154.02, 138.78, 138.43, 136.81, 136.55, 134.29, 126.55, 114.59, 113.01, 111.99, 111.95, 82.96, 80.11, 80.07, 69.13, 68.97, 65.95, 65.93, 64.72, 55.67, 55.54, 55.11, 55.05, 44.20, 44.14, 43.78, 43.48, 40.60, 40.44, 37.98, 37.70, 31.96, 29.76, 29.73, 29.69, 29.60, 27.51, 27.29, 26.59, 26.55, 24.61, 23.68, 23.29, 22.72, 22.08, 14.16, 13.47.

5.1.9 General procedure for preparing of 3-(((8S,9S,13S,14S,17S)-(R,E)-2-hydroxy-3-oxo-5-(4-(trifluoromethyl)phenyl)pent-4-en-2-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-yl)oxy)propane-1,2-diyl dinitrate (29)

To a solution of \(\alpha\)-hydroxy methyl ketone 28 (0.1836 g, 0.4 mmol) in THF (1 mL) in an open 20 mL-glass vial, 4-(trifluoromethyl)benzaldehyde (0.8 mmol) and sodium hydroxide (0.0464 g, 1.16 mmol) were added at room temperature and the reaction mixture was then heated at 105 °C for 10 min with continuing addition of THF by an approximate rate of 0.5 mL/min (avoid dryness). The reaction mixture was then quenched by the addition of water (15 mL), followed by extraction with EtOAc (3x20 mL), washed with brine, dried over anhydrous Na\(_2\)SO\(_4\), and concentrated in vacuo [37, 62-64]. The resulting yellow oil was purified by flash chromatography (Hexane/EtOAc, 8:2) to yield the title product NO-CIEA 29 in 44%.

Yellow solid; \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) 7.75 (d, \(J = 15.5 \text{ Hz}, 1\text{H}\)), 7.63 (d, \(J = 8.6 \text{ Hz}, 2\text{H}\)), 7.59 (d, \(J = 8.6 \text{ Hz}, 2\text{H}\)), 7.14 (d, \(J = 8.6 \text{ Hz}, 1\text{H}\)), 7.04 (d, \(J = 15.5 \text{ Hz}, 1\text{H}\)), 6.62 (dd, \(J = 8.6, 2.5 \text{ Hz}, 1\text{H}\)), 6.54 (d, \(J = 2.5 \text{ Hz}, 1\text{H}\)), 5.53 – 5.46 (m, 1H), 4.83, 4.69 (two dd, \(J = 12.9, 3.3, 12.9, 6.6 \text{ Hz}, 2\text{H}\)), 4.15 – 4.13, 4.12 – 4.10 (two m, 2H), 4.00 (s, 1H), 2.80 – 2.69 (m, 2H), 2.30 – 2.05 (m, 3H), 2.03 – 1.86 (m, 1H), 1.82 – 1.79 (m, 2H), 1.61 – 1.51 (m, 3H), 1.49 (s, 3H), 1.36 – 1.15 (m, 5H), 0.89 (s, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 201.72, 155.45, 143.67, 138.46, 137.68, 134.29, 128.77, 128.73, 126.56, 125.95, 120.81, 114.61, 111.96, 79.38, 68.96, 64.70,
55.67, 55.04, 44.36, 43.81, 40.63, 38.02, 29.75, 27.50, 26.62, 26.28, 24.27, 23.65, 22.07, 13.65. HRESI-MS m/z calcd for [M+H]⁺ C₃₃H₃₈F₃N₂O₉: 663.252392, found: 663.255249.

5.1.10 General procedure for preparing of (R,E)-2-(((S,S,S,S,S,S,S,S,S,S)-3-(allyloxy)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-2,6-dihydroxy-6-methylhept-4-en-3-one (31)

To a stirred solution of α-hydroxy methyl ketone 27 (0.500 g, 0.79 mmol) in THF (1.6 mL), LDA (1.42 mL, 2.83 mmol, 2 M in THF) was added at -78 °C and stirred for 1 h. A solution of 2-((tert-butyldimethylsilyl)oxy)-2-methylpropanal 11 (0.3997 g, 1.98 mmol) in THF (5.3 mL) was then added at -78 °C and the reaction mixture was allowed to slowly warm to room temperature and stirred for 20 h. The reaction mixture was then quenched by the addition of saturated NH₄Cl (20 mL), extracted with EtOAc (3x50 mL), dried over anhydrous Na₂SO₄, and concentrated in vacuo [37, 63]. The crude product was purified by silica gel column chromatography with hexanes/EtOAc (9.5:0.5) to yield compounds 26 and 31.

31; White solid; 25% yield; ¹H NMR (600 MHz, CDCl₃) δ 7.07 (d, J = 8.6 Hz, 1H), 7.03 (d, J = 15.2 Hz, 1H), 6.60 (dd, J = 8.6, 2.7 Hz, 1H), 6.57 (d, J = 15.2 Hz, 1H), 6.52 (d, J = 2.7 Hz, 1H), 5.93 (ddt, J = 17.2, 10.6, 5.3 Hz, 1H), 5.28 (dd, J = 17.2, 1.6 Hz, 1H), 5.15 (dd, J = 10.5, 1.6 Hz, 1H), 4.39 (dt, J = 5.3, 1.6 Hz, 2H), 4.00 (s, 1H), 2.77 – 2.66 (m, 2H), 2.21 – 2.05 (m, 3H), 1.76 – 1.68 (m, 2H), 1.58 (s, 1H), 1.56 – 1.53 (m, 1H), 1.48 – 1.41 (m, 3H), 1.37 (s, 3H), 1.29 (s, 6H), 1.24 – 1.07 (m, 5H), 0.82 (s, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 202.37, 156.47, 156.00 (CO-CH=CH), 137.97, 133.62, 132.90, 126.23, 118.10 (CO-CH=CH), 117.47, 114.72, 112.22, 79.20, 71.37 (C24-OH), 68.81, 55.66, 54.62, 44.32, 43.84, 40.60, 38.10, 29.83, 29.57 (C25 & C26), 27.63, 26.62, 24.17, 23.67, 21.98, 13.65. ¹³C DEPT-NMR (151 MHz, CDCl₃) δ 156.01, 133.62, 126.23, 118.10, 117.47 (inverted CH₂), 114.73, 112.23, 68.81 (inverted CH₂), 55.66, 54.63, 43.84, 40.60 (inverted CH₂), 38.10, 29.83 (inverted CH₂), 29.56, 27.63 (inverted CH₂), 26.62 (inverted CH₂), 24.17, 23.67 (inverted CH₂), 21.99 (inverted CH₂), 13.66.
6 Biological evaluation

6.1 In vitro cytotoxicity assay

The in vitro antiproliferation of the chemical compounds was measured by the MTT reagent, as described in the literature [42-44]. Briefly, \(2 \times 10^4\) HepG2 cells in 100 \(\mu\)L of medium per well were seeded in 96-well plates. After cultured for 24 h in 37 °C humidified incubator (5% CO\(_2\)), cells were incubated in complete mediums with the Erlotinib (as a reference drug), absence (negative control) and presence of various concentrations of tested compounds, respectively, for 48 h. Each group was arranged four parallel wells. MTT solution (20 \(\mu\)L; 5 mg/mL) was added to each well and incubated at 37 °C for 2 h. Last, the MTT-containing medium was discarded and 200 \(\mu\)L of DMSO per well was added and mixed well to dissolve the formazan crystals newly formed. Absorbance of each well was determined by a microplate reader (Hidex Sense) at a 570 nm wavelength. Survival percentage was calculated using the following equation:

\[
\text{Inhibitory rate } \% = \frac{(\text{Abs}_{570 \text{ control cells}} - \text{Abs}_{570 \text{ treated cells}})}{\text{Abs}_{570 \text{ control cells}}} \times 100.
\]

IC\(_{50}\) values were obtained from linear regression analysis of the concentration-response curves plotted for each tested compound.

6.2 Measurement of Intracellular NO

The intracellular level of NO after compounds treatment was quantified using the NO-sensitive fluorophore DAF-FM DA. HepG2 cells were seeded in a six-well plate at 7.5x10\(^5\) cells per well and allowed to grow overnight. Cells were loaded with 2 mL of 2.5 \(\mu\)M DAF-FM DA in HBSS in each well at 37 °C and 5% CO\(_2\). After 30 min of incubation, the cells were rinsed with HBSS two times to remove excess of probe. The compounds were dissolved in DMSO and different concentrations were prepared and added to the cells. After 1, 6, 12 and 24 h of incubation, cells were washed with HPSS, trypsinized and pipetted into flow cytometer test tubes. The fluorescence of the benzotriazole derivative formed on DAF-FM DA’s reaction with NO was analyzed by flow cytometer (BD Acuuri C6, Becton-Dickinson, Mountain View, CA) with the excitation source at 495 nm and emission at 515 nm. Data are expressed as mean values of at least two runs ± the standard deviation (SD) [43, 50-52].
6.3 Cell cycle analysis

The cells were seeded as 2.5x10^5 cells/mL in a 6-well plate (2 mL/well) and allowed to adhere overnight at 37 °C and 5% CO_2. The cells were incubated with NO-CIEA 17 (4.69 μM), 20a (12.5 μM) and DMSO (0.001%) as a control for 24 h. The cells were washed twice with ice-cold 1X PBS (HyClone™ Laboratories, Inc) and collected after trypsinization [69]. The cell pellet was washed two times with ice-cold 1X PBS and fixed with ice-cold 70% ethanol overnight at -20 °C. After that, the cells were washed once with ice-cold PBS and the second wash with ice-cold PBS-2% FBS. The cell pellet was re-suspended in 500 μL propidium iodide (PI)/RNase staining solution (BD Biosciences) and 0.1% triton x-100 for 30 min. at room temperature in the dark and analyzed within 1 h by flow cytometer (BD Accuri C6, Becton-Dickinson, Mountain View, CA). Data was analyzed by MFLT32 software and 10,000 events with slow flow rate were recorded for each sample [32, 70].

6.4 In-cell Western assay (ICW)

HepG2 Cell line were seeded into clear bottomed and black walled 96-well plates with a density of 0.5x10^6 cells per mL and allowed to grow to confluence. The next day, cells were treated with different concentrations of NO-CIEA 17 (1.2, 2.4 and 4.8 μM) for 24 h and DMSO was used as a control. Cells were then fixed with 3.7% formaldehyde in 1X PBS for 30 min. and then washed and permeabilized with 0.1% TritonX-100 in 1X PBS. Cells were then blocked with 1X PBS fish gel solution and then incubated with an antibody to EGFR (cell signaling technology), Phospho-EGFR (Santa Cruz Biotechnology), MAPK (Erk1/2; Santa Cruz Biotechnology), Phospho-MAPK (p-Erk1/2; Santa Cruz Biotechnology), Phospho-MEK1/2 (Cell Signaling Technology), β-actin (Invitrogen, Thermo Fisher Scientific), MRP2 (Abcam) and GAPDH (Santa Cruz Biotechnology) for 2 h with gentle shaking then incubated overnight at 4 °C without shaking (i.e: stationary). Cells were then washed with 0.1% Tween-20 in 1X PBS four times and then incubated with secondary antibodies conjugated to IRdye for 1 h with gentle shaking (protect from light). Cells were then washed with 0.1% Tween-20 in 1X PBS four times. After the last wash, any residual liquid was gently pipetted out and the plate was blotted dry using the In-cell western protocol on an Odyssey® imager (LI-COR®) according to manufacturer’s directions [53-57]. Phospho-proteins were normalized for total protein signals while p-MEK1/2 and MRP2 were normalized for the β-actin and GAPDH loading controls.
respectively, to correct for well-to-well variation in cell number and percent-inhibition determined relative to control wells. Data are expressed as mean values of at least two runs ± the standard deviation (SD).

6.5 Docking studies

This was done using OpenEye® molecular Modeling software. A virtual library of structurally modified NO-CIEAs were energy minimized using MMFF94 force field, followed by generation of multi-conformers using OMEGA® application. The whole energy minimized library will be docked along with the prepared EGFR (PDB code: 1M17) or ERK (PDB code: 2OJJ) using FRED® application to generate a physical property (ΔG) reflecting the predicted energy profile of ligand-receptor complex. Vida® application can be employed as a visualization tool to show the potential binding interactions of the ligands to the receptor of interest [32, 71-74].

7 Acknowledgments

We wish to acknowledge the Egyptian government for financial support via the Egyptian Ministry of Higher Education and Scientific Research. We are also grateful to OpenEye® molecular modeling software for supporting an academic license.

8 References


Table 1. Consensus scores of NO/CIEA hybrids and Erlotinib and 82A as reference drugs.

Table 2. Anti-proliferative activity of NO-CIEAs 17, 20a-g, 23a,b, 25, 28 and 30 towards HepG2 and HepG2-R cell lines

Table 3. Anti-proliferative activity of compound 13 and des-NO-CIEA 16 towards HepG2 and HepG2-R cell lines

Figure 1. Structures of NCX-1000, CUCs and current antitumor drugs.

Figure 2. Design strategy for target compounds NO/CIEA hybrids.

Figure 3. Visual representation for A) Erlotinib; B) NO-CIEA 17; C) NO-CIEA 20f and; D) overlay of the binding modes of NO-CIEAs 17 (gray) and 20f (green) docked with PDB: 1M17. These compounds illustrated HB (dashed lines) and hydrophobic-hydrophobic interaction towards EGFR ATP-binding site.

Figure 4. Visual representation for A) standard 82A ligand and; B) NO-CIEA 17 docked with PDB: 2OJJ. The compounds exhibited HB (dashed lines) and hydrophobic-hydrophobic interaction towards glycine-rich loop of ERK.

Figure 5. Intracellular NO release in HepG2 and HepG2-R cells as measured by DAF-FM DA assay. a) JS-K (reference drug); b) NO-CIEA 17 against HepG2 cells and; c) NO-CIEA 20a against HepG2 cells d) NO-CIEA 17 against HepG2-R cells. Values reported are averages ± SD of at least two measurements.

Figure 6. Flow cytometric analysis of cell cycle parameters of HepG2 cells incubated for 24 h; a) control; b) 4.69 µM (IC<sub>50</sub>) of NO-CIEA 17; c) 12.5 µM (IC<sub>50</sub>) of NO-CIEA 20a.

Figure 7. In-Cell Western analysis of predicted protein targets of NO-CIEA 17. A) Analysis of EGFR phosphorylation in HepG2 cells; ICW plate image and p-EGFR intensity in 800 channel was normalized to total EGFR intensity in 700 channel. B) Analysis of ERK1/2 phosphorylation in HepG2 cells; ICW plate image and p-ERK1/2 intensity in 800 channel was normalized to total ERK1/2 intensity in 700 channel. C) Analysis of MEK1/2 phosphorylation in HepG2 cells; ICW
plate image and p-MEK1/2 intensity in 700 channel was normalized to β-actin intensity in 800 channel. D) Analysis of MRP2 inhibition in HepG2-R cells; ICW plate image and MRP2 intensity in 800 channel was normalized to GAPDH intensity in 700 channel. Values reported are averages ± SD of at least two measurements.

**Scheme 1:** Synthesis of key intermediates α-hydroxy methyl ketones; i) TBSCl, imidazole, DMF, overnight, rt; ii) t-BuOK, TEPB, THF, CH₃CH=PPh₃, 1 h, reflux (80 °C), 6-7h; iii) 9-BBN, THF, 60 °C, 10% NaOH, H₂O₂, rt, 2 h; iv) PCC, NaOAc, 4Å MS, DCM, rt; v) TMSCN, ZnI₂, dry DCM, rt; vi) MeLi, Et₂O, 0 °C.

**Scheme 2:** Synthesis of protected methylpropanal; i) TBSCl, imidazole, DMF, rt, 3 days; ii) DIBAL-H, Rochale salt, THF, -78 °C; iii) condition A: TPAP, NMO, 4Å MS, 12 h; condition B: PCC, NaOAc, dry DCM, 4Å, 12 h.

**Scheme 3:** Synthesis of NO-releasing furoxan moiety; i) NaNO₂, HOAc, -10 °C, 24h; ii) MsCl, TEA, dry DCM, 0 °C to rt, 2 h.

**Scheme 4:** Synthesis of (19R)-NO-CIEA 17 with CUCs side chain; i) LDA, THF, -78 °C, 1 h, 11, -78 °C to rt, 20 h; ii) TBAF, THF, rt, 12 h; iii) NaOH, THF, 14, reflux, 2 h.

**Scheme 5:** Synthesis of (19R)-NO-CIEAs 20a-g with aromatic and heterocyclic side chains; i) ArCHO, NaOH, THF, 105 °C, 10-30 min.; ii) TBAF, THF, rt, 12 h; iii) 14, NaOH, THF, reflux, 2 h.

**Scheme 6:** Synthesis of (19S)-NO-CIEAs 23a,b with aromatic side chain; i) ArCHO, NaOH, THF, 105 °C, 10-30 min.; ii) TBAF, THF, rt, 12 h; iii) 14, NaOH, THF, reflux, 2 h.

**Scheme 7:** Synthesis of iodonitrate intermediate 27 and nitrate/CIEA hybrid 29; i) TBAF, THF, rt, 12 h; ii) 14, NaOH, THF, reflux, 2 h; iii) allyl bromide, NaOH, THF, reflux, 2 h; iv) I₂ (1-3 eq.), CH₃CN, rt to reflux, 0.5-1 h; v) I₂ (1 eq.), CH₃CN (3 eq.), AgNO₃ (3 eq.), 30 min, rt; vi) AgNO₃ (3 eq.), CH₃CN, reflux, 7 h; vii) p-CF₃PhCHO, NaOH, THF, 105 °C, 10 min.

**Scheme 8:** Unexpected products of aldol condensation with iodonitrate intermediate 27; i) LDA, THF, -78 °C, 1h, 11, 20 h, rt; ii) p-methoxybenzaldehyde, NaOH, THF, reflux, 1h.
Table 1. Consensus scores of NO/CIEA hybrids and Erlotinib and 82A as reference drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Consensus score</th>
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<td></td>
<td>EGFR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ERK&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>20a</td>
<td>18</td>
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<tr>
<td>20c</td>
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<td>42</td>
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<tr>
<td>20d</td>
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<tr>
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<sup>a</sup> EGFR co-crystalized ligand, <sup>b</sup> ERK1 co-crystalized ligand, <sup>c</sup> PDB code: 1M17, <sup>d</sup> PDB code: 2OJJ
**Table 2.** Anti-proliferative activity of NO-CIEAs 17, 20a-g, 23a,b, 25, 28 and 30 towards HepG2 and HepG2-R cell lines

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<tr>
<th>Compound No.</th>
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<th>IC$_{50}$ (µM)$^b$</th>
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<td>17.84 ± 1.58</td>
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$^a$ positive control, $^b$ IC$_{50}$: concentration of the test compound that inhibits 50% of cell growth (results are expressed as the mean ± SD (n = 3), $^c$ Calculated by Molinspiration, $^d$ Molecular polar surface area
Table 3. Anti-proliferative activity of compound 13 and des-NO-CIEA 16 towards HepG2 and HepG2-R cell lines

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>IC$_{50}$ (µM)$^b$</th>
<th>ClogP$^c$</th>
<th>PSA$^{c,d}$</th>
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$^a$ positive control, $^b$ IC$_{50}$: concentration of the test compound that inhibits 50% of cell growth (results are expressed as the mean ± SD (n = 3), $^c$ Calculated by Molinspiration [75], $^d$ Molecular polar surface area
Figure 1. Structures of NCX-1000, CUCs and current antitumor drugs.

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**B**

**Conc. (µM)**

<table>
<thead>
<tr>
<th>Control</th>
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<th>2.35</th>
<th>4.69</th>
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</table>

**Overlay**

**ERK1/2**

**p-ERK1/2**

**% p-ERK1/2 related to ERK1/2**

![Graph](image1)

**C**

**Conc. (µM)**

<table>
<thead>
<tr>
<th>Control</th>
<th>1.17</th>
<th>2.35</th>
<th>4.69</th>
</tr>
</thead>
</table>

**Overlay**

**β-Actin**

**p-MEK1/2**

**% p-MEK1/2 related to β-actin**

![Graph](image2)
Figure 7. In-Cell Western analysis of predicted protein targets of NO-CIEA 17. A) Analysis of EGFR phosphorylation in HepG2 cells; ICW plate image and p-EGFR intensity in 800 channel was normalized to total EGFR intensity in 700 channel. B) Analysis of ERK1/2 phosphorylation in HepG2 cells; ICW plate image and p-ERK1/2 intensity in 800 channel was normalized to total ERK1/2 intensity in 700 channel. C) Analysis of MEK1/2 phosphorylation in HepG2 cells; ICW plate image and p-MEK1/2 intensity in 700 channel was normalized to β-actin intensity in 800 channel. D) Analysis of MRP2 inhibition in HepG2-R cells; ICW plate image and MRP2 intensity in 800 channel was normalized to GAPDH intensity in 700 channel. Values reported are averages ± SD of at least two measurements.
Scheme 1: Synthesis of key intermediates α-hydroxy methyl ketones; i) TBSCl, imidazole, DMF, overnight, rt; ii) t-BuOK, TEPB, THF, CH₃CH=PP₃, 1 h, reflux (80 °C), 6-7h; iii) 9-BBN, THF, 60 °C, 10% NaOH, H₂O₂, rt, 2 h; iv) PCC, NaOAc, 4Å MS, DCM, rt; v) TMSCN, ZnI₂, dry DCM, rt; vi) MeLi, Et₂O, 0 °C.

Scheme 2: Synthesis of protected methylpropanal; i) TBSCl, imidazole, DMF, rt, 3 days; ii) DIBAL-H, Rochale salt, THF, -78 °C; iii) condition A: TPAP, NMO, 4Å MS, 12 h; condition B: PCC, NaOAc, dry DCM, 4Å, 12 h.

Scheme 3: Synthesis of NO-releasing furoxan moiety; i) NaNO₂, HOAc, -10 °C, 24 h; ii) MsCl, TEA, dry DCM, 0 °C to rt, 2 h.
Scheme 4: Synthesis of (19R)-NO-CIEA 17 with CUCs side chain; i) LDA, THF, -78 °C, 1 h, 11, -78 °C to rt, 20 h; ii) TBAF, THF, rt, 12 h; iii) NaOH, THF, 14, reflux, 2 h.
Scheme 5: Synthesis of (19R)-NO-CIEAs 20a-g with aromatic and heterocyclic side chains; i) ArCHO, NaOH, THF, 105 °C, 10-30 min.; ii) TBAF, THF, rt, 12 h; iii) 14, NaOH, THF, reflux, 2 h.
Scheme 6: Synthesis of (19S)-NO-CIEAs 23a,b with aromatic side chain; i) ArCHO, NaOH, THF, 105 °C, 10-30 min.; ii) TBAF, THF, rt, 12 h; iii) 14, NaOH, THF, reflux, 2 h.
Scheme 7: Synthesis of iodonitrate intermediate 27 and nitrate/CIEA hybrid 29; i) TBAF, THF, rt, 12 h; ii) 14, NaOH, THF, reflux, 2 h; iii) allyl bromide, NaOH, THF, reflux, 2 h; iv) I$_2$ (1-3 eq.), CH$_3$CN, rt to reflux, 0.5-1 h; v) I$_2$ (1 eq.), CH$_3$CN (3 eq.), AgNO$_3$ (3 eq.), 30 min, rt; vi) AgNO$_3$ (3 eq.), CH$_3$CN, reflux, 7 h; vii) p-CF$_3$PhCHO, NaOH, THF, 105 °C, 10 min.
Scheme 8: Unexpected products of aldol condensation with iodonitrate intermediate 27; i) LDA, THF, -78 °C, 1h, 11, 20 h, rt; ii) p-methoxybenzaldehyde, NaOH, THF, reflux, 1h.
Graphical abstract

Estrone → NO-CIEA 17

Broad spectrum anti-proliferative activity
IC₅₀ against HepG2 cell line = 4.69 ± 0.48 mM
IC₅₀ against HepG2-R cell line = 8.21 ± 0.73 mM
Highlights:

1) Design and synthesis of a novel series of nitric oxide donating Cucurbitacin Inspired Estrone Analogs (NO-CIEAs).

2) Binding affinity of NO-CIEA 17 and 20a towards EGFR and ERK was suggested by docking study.

3) Anti-proliferative activity was examined against HepG2 and Erlotinib-resistant HepG2 cell lines.

4) Intracellular NO release was examined using NO-sensitive fluorophore DAF-FM DA.

5) Flow cytometric studies showed that both 17 and 20a mainly arrested the HepG2 cells in the G0/G1 phase.

6) ICW assay revealed the dual inhibitory activity of NO-CIEA 17 towards EGFR and ERK in a dose dependent manner.

7) The significant impact on the chemotherapeutic resistance was confirmed by inhibition of MRP2 in a dose dependent manner by NO-CIEA 17.