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PII: S0960-894X(19)30856-X
DOI: https://doi.org/10.1016/j.bmcl.2019.126878
Reference: BMCL 126878

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 28 October 2019
Accepted Date: 28 November 2019

Please cite this article as: Hossain, M., Das, S., Das, U., Doroudi, A., Zhu, J., Dimmock, J.R., Novel hybrid molecules of 3,5-bis(benzylidene)-4-piperidones and dichloroacetic acid which demonstrate potent tumour-selective cytotoxicity, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.126878

Novel hybrid molecules of 3,5-bis(benzylidene)-4-piperidones and dichloroacetic acid which demonstrate potent tumour-selective cytotoxicity

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ABSTRACT

A novel class of hybrid molecules **2a-o** was designed as candidate antineoplastic agents from dichloroacetic acid which is a known inhibitor of pyruvate dehydrogenase kinase and a number of cytotoxic 3,5-bis(benzylidene)-4-piperidones **1**. In general these new hybrid molecules are potent cytotoxins towards human HCT116 colon cancer cells. A number of lead molecules were emerged having the IC_{50} values in the double digit nanomolar range. Most of these compounds are less toxic to human CRL1790 non-malignant colon cells and hence the selectivity index (SI) figures for most of the compounds are huge; in the case of **2c-g**, **m, n**, the SI values are in excess of 100. Compounds **2g**, **2j**, **2m** and **2n** displayed more than 100-fold higher potency than the reference drug 5-FU. Quantitative structure-activity relationships revealed that the potencies of the compounds in series **2** increase as the magnitude of the Hammett σ and Taft σ* values rise. X-ray crystallographic of a representative compound **2c** revealed various structural features which may influence cytotoxic potencies. Several representative compounds lowered the mitochondrial membrane potential and increased the production of reactive oxygen species in HCT116 cells. A minimal effect was noted in altering the percentage of cells in different phases of the cell cycle. Some future directions have been outlined for analog development.

Keywords : cytotoxicity / 3,5-bis(benzylidene)-4-piperidones / tumour-selective toxicity / cell cycle analysis / mitochondrial membrane potential / X-ray crystallography / dichloroacetyl / QSAR

Cancer is a devastating disease globally. The primary interest of this laboratory is the discovery of novel cytotoxins which display high potency and have greater toxicity towards neoplasms than non-malignant cells. We have demonstrated previously that many 3,5-bis(benzylidene)-4-piperidones **1** possess promising cytotoxic potencies.[1-3] These compounds are believed to react readily with thiols but not amino or hydroxyl groups.[4-6] Thus these molecules should not interact with nucleic acids and the problem of causing genotoxicity may therefore be absent in these compounds. In addition, these molecules permit sequential interactions with cellular constituents whereby reactions occur at one of the olefinic carbon atoms which is followed by further thiol alkylation at the remaining olefinic carbon atom. This sequence of reactions may lead to compounds which display greater toxicity to tumours than normal cells since previous studies revealed that after an initial chemical insult, certain tumours become more susceptible to cytotoxins than non-malignant cells.[7,8] These interesting structural features associated with **1** has created our further interest to design novel molecules based on 3,5-bis(benzylidene)-4-piperidone as the core cytotoxic warhead to enhance tumour-selective cytotoxicity.

A number of tumours have undergone metabolic reprogramming using glycolysis to produce energy instead of oxidative phosphorylation. This phenomenon is known as the Warburg effect. Pyruvate dehydrogenase kinase 1 (PDK1) is required for glycolysis and inhibiting this enzyme will therefore reverse the Warburg effect. Dichloroacetic acid (DCA) is an inhibitor of PDK1[9] that has been evaluated as an anticancer agent.[10] However, there are problems associated with the clinical use of DCA such as its low potency. Thus the objective of the present study was to attach the dichloroacetyl group to the cytotoxic warhead **1** to create novel hybrid molecules as potent cytotoxic agents. In addition, depending on the lability of the bond between DCA and the cytotoxins, release of DCA may take place and since DCA has demonstrated chemosensitizing properties,[11] the potency of the cytotoxin may be enhanced.

In this study, novel hydride molecules in series **2** have been disclosed as potent cytotoxic agents. The aryl substituents in **2** were mainly chosen on the basis of their varied electronic, hydrophobic and steric properties. For example, substituents are found in all four quadrants of a Craig diagram for *para* substituents in regard to their electronic and hydrophobic properties.[12] In addition, two related compounds were prepared namely **3** and **4** in order to develop structure-activity relationships *vide infra*. Since colon cancer is one of our main research interests, the compounds were evaluated against human HCT116 colon cancer cells which were used previously in our studies with conjugated unsaturated ketones.[1,13,14] In order to assess tumour-selective cytotoxicity, these molecules were screened against non-malignant human CRL1790 colon epithelial cells.

[PLEASE INSERT SCHEME 1]

The synthesis of the compounds in series **1-4** has been portrayed in Schemes 1 and 2. In brief, the compounds in series **2** were prepared as follows. Acid catalyzed condensation between various aryl aldehydes and 4-piperidone led to the formation of **1a-o** which were further reacted with dichloroacetyl chloride to produce compounds in series **2** (Scheme 1). The synthesis of **3** was obtained by *N*-acetylation of **1a** while compound **4** was prepared from **1l** (Scheme 2). Details of the syntheses are given in the supplemental section.

[PLEASE INSERT SCHEME 2]

The evaluation of **1a**, **2a-o**, **3** and **4** as well as two reference compounds namely sodium dichloroacetate and 5-fluorouracil (5-FU) towards HCT116 and CRL1790 cells are portrayed in Table 1.

[PLEASE INSERT TABLE 1]

The evaluation of series **2** against HCT116 cells reveals that with the exception of **2l**, **o**, all of the analogs in series **2** have submicromolar IC_{50} values, i.e., in 87 % of the compounds. The most potent molecules with IC_{50} values in the 30-60 nanomolar range are **2d**, **e**, **g**, **j**, **m**, **n** and are clearly lead molecules. On the other hand, the IC_{50} values of **2l**, **o** are in the low micromolar range. Both of these compounds have electron releasing groups in the aryl rings, i.e., the σ_p values of the 4-hydroxy and 4-dimethylamino groups are -0.37 and –0.83, respectively.[15] This effect will increase the electron densities on the olefinic carbon atoms thereby retarding nucleophilic attack by cellular thiols. Methylation of the hydroxyl groups led to **2h** with an IC_{50} value which is 14 times lower than **2l**.

The question arises as to whether one or more physicochemical properties of the aryl substituents influence cytotoxic potencies. The sigma (σ) and sigma star (σ*) constants are a measure of the electronic properties of the aryl substituents while the pi (π) and molar refractivity (MR) figures reflect the hydrophobic and steric properties of the groups in the aryl rings, respectively. Hence linear and semilogarithmic plots between each of these parameters and the IC_{50} values towards HCT116 and CRL1790 cells were made. For HCT116 cells, negative correlations were noted between the σ/σ* constants and the IC_{50} values of **2a-o** from the linear ($p<0.01$) and semilogarithmic ($p<0.01$) plots. When the outlier **2l** was omitted, a negative correlation was found between the IC_{50} figures of **2a-k**, **m-o** and the σ/σ* constants from the linear ($p<0.05$) and the semilogarithmic ($p<0.01$) plots. The outlier **2o** was then removed from the analysis. A negative correlation between the IC_{50} values of **2a-n** and the σ/σ* constants were observed from the semilogarithmic plot ($p<0.05$). No other correlations were noted. These data reveal that potency increases as the magnitude of the σ/σ* constants rise. Hence in the future compounds with strongly electron attracting groups should be placed in the aryl rings. Linear and semilogarithmic plots between the IC_{50} values of **2a-o**, **2a-k**,**m-o** and **2a-n** towards CRL1790 cells and the σ/σ*, π and MR constants of the aryl substituents were made. Only a trend to a correlation between the σ/σ*values and the IC_{50} figures of **2a-n** in the linear and semilogarithmic plots were noted ($p<0.1$).

A comparison of the cytotoxic potencies of **1a**, **2a** and **3** were made with a view to finding if the presence of a dichloroacetyl group contributes significantly to potency. The IC_{50} figures of **1a, 2a** and **3** are 3.50, 0.23 and 0.28 μM, respectively, which indicates that while *N*-acylation of **1a** leads to potency increases, the introduction of two chloro atoms into **3** creating **2a** does not lower the IC_{50} figures.

Compound **4** has a *N*-acyl group on the piperidyl nitrogen atom and dichloroacetyloxy substituent on both aryl rings. This compound has the same potency towards HCT116 cells as **2a** and **3** suggesting that the common structure of **2a**, **3** and **4** namely the 3,5-bis(benzylidene)-1-carbonyl-4-oxo-piperidinyl group affects cytotoxic potencies considerably.

The biodata generated for **1a**, **2a-o**, **3** and **4** were compared with the potency of 5-FU which is a drug used in treating colon cancer. With the exception of **2l, o**, all of the compounds are more potent than 5-FU, e.g., **2g, j, 2m**, **n** are >100 times more potent than 5-FU towards

HCT116 cells. As indicated in Table 1, a second reference compound namely sodium dichloroacetate displays weak potency towards HCT116 cells.

The next consideration was to determine whether **1a**, **2a-o**, **3** and **4** are less toxic to human CRL1790 non-malignant colon cells. These results are presented in Table 1. Except for **2o**, the IC_{50} values are greater towards CRL1790 cells than HCT116 cells indicating that these compounds display greater toxicity to the tumour cell line. In order to determine the magnitude of the differential in toxicity towards the cell lines, selectivity index (SI) values were computed. These figures are the quotients of the IC_{50} values of the compounds towards CRL1790 and HCT116 cell lines. These SI values are presented in Table 1. The majority of the SI values are huge. The greatest selective toxicity was noted with the following compounds (SI values in parentheses) namely **2m** (540), **2j** (378), **2n** (378) and **2g** (305). Virtually all of the compounds have SI values greater than 5-FU while sodium dichloroacetate has the same toxicity to HCT116 and CRL1790 cells.

[PLEASE INSERT FIGURE 1]

X-ray crystallographic structure of a representative compound **2c** was determined with a view to finding some of its molecular features which may contribute to its cytotoxic potency. The ORTEP diagram of **2c** is presented in Figure 1. An interesting feature of the X-ray crystallographic structure of this compound is that it is disordered. The fluoro atom is attached to the C9 carbon atom in 81 % of the unit cells while in 19% of the cases, the fluoro atom is bonded to the C11 atom. Cellular thiols are predicted to undergo nucleophilic attack at carbon atoms C6 and C13. Hence the stereochemistry at this portion of the molecule may affect the rate and extent of thiol alkylation. This molecule adopts the *E* configuration at the two olefinic double bonds.An evaluation was made to determine whether the two aryl rings are coplanar with the adjacent olefinic groups. The C2-C13-C14-C15 and C2-C13-C14-C19 torsion angles are 148.85 and -32.14, respectively. The C4-C6-C7-C8 and C4-C6-C7-C12 angles are -157.63 and 25.41, respectively. Hence the aryl rings do not lie in the same plane as the olefinic groups and the two rings are orientated in different directions. This lack of coplanarity may be due to non-bonded interactions between the C1He and C19H atoms as well as the C5He and C12H atoms. The interatomic distances are 2.318 Å and 2.206 Å, respectively, indicating that non-bonded interactions occurred.These observations may contribute to the relative potencies of **2c** towards HCT116 and CRL1790 cells which give rise to the huge SI value of 135. In other words, the stereochemistry of **2c** (and analogs) may be such as to render the compound highly toxic to the neoplasm but much less so to non-malignant cells. The ORTEP diagram suggests that the C5He and Cl1 atoms are in close proximity but the interatomic distance is 2.817 Å revealing the absence of non-bonded interactions. On the other hand, the C5He and C12H atoms are 2.206 Å apart suggesting some non-bonded interaction may be taking place. In addition, the conformation of the central piperidine ring may affect the alignment of the molecule at binding sites and in the case of **2c** it is in the half chair conformation.

The next stage of the investigation was to attempt to find some of the ways in which representative compounds exert their toxicity to HCT 116 cells. Four compounds were chosen for these evaluations namely **2a**, **o**, **n** and **3** which differ in their potencies (**2n**>**2a**, **3**>**2o**) and tumour-selective cytotoxicity (**2n**>**3**>**2a**>**2o**). Three mode of action studies were undertaken in HCT116 cells by examining whether these compounds lower the mitochondrial membrane

potential (MMP), increase the production of reactive oxygen species (ROS) and have any effect on the cell cycle.

[FIGURE 2]

ATP production is controlled by the mitochondria. In many tumours, the MMP is higher than is found in non-malignant cells.[16] Hence the disruption of the MMP in neoplasms may lead to a reduction in cell growth or apoptosis. Concentrations of the IC_{50} and twice the IC_{50} values of **2a**, **n**, **o** and **3** towards HCT 116 cells were used and the results are portrayed in Figure 2. The data indicate that in the case of **2a**, **n** and **3** as well as 5-FU, cytotoxicity is caused, at least in part, by a lowering of the MMP. On the other hand, the toxic effects of **2o** are not likely due to any effect on the MMP.

[FIGURE 3]

Cancer cells show redox imbalance due to increased ROS level compared to normal cells. This unique feature of cancer cells is exploited for targeted cancer therapy. In order to assess the effect of these novel cytotoxins on ROS generation, compounds **2a**, **n**, **o** and **3** were evaluated in HCT 116 cells. The results reveal that these compounds and 5-FU increase the concentration of ROS which likely contributes to their toxicity. These results are portrayed in Figure 3.

[TABLE 2]

A number of anticancer drugs act at different stages of the cell cycle. Hence **2a**, **n**, **o**, **3** were examined for this effect in HCT116 cells. The results are presented in Table 2. In general the biodata indicate that these compounds have little or no effect on different stages of the cell cycle. There is a slight elevation in the percentage of cells undergoing apoptosis and in general a lowering of cells in the G_0-G_1 phase.

A question arises as to whether a rupture of the amidic bond occurs during the bioevaluation of the compounds in series **2**. Hence **2a** was incubated at 37˚C in a mixture of dimethylsulfoxide and 0.1 M phosphate buffer.[17] The mixture was extracted with chloroform after 0, 24 and 48 hours and the product was examined by ^{1}H NMR spectroscopy. The three spectra are identical indicating that under these conditions **2a** was stable at 37˚C for 48 hours (the temperature and time of the cytotoxic bioassays). Hence the bioactivity displayed by **2a**, and probably the related compounds **2b**-**o** are likely due to the intact molecules and not to any breakdown products.

Finally investigation was to assess druglikeness properties of **2a**-**o** as per the guidelines suggested by Lipinski et al.[18] and Muegge et al.[19] using a free web tool, SwissADME.[20] The physicochemical data reveal that all the compounds except **2g** comply to the criteria for druglike properties (data not shown), thereby confirming that compounds in series **2** are excellent cytotoxins that warrant further preclinical evaluations as candidate antineoplstic agents.

In conclusion, a novel series of antineoplastic agents have been disclosed which display far greater toxicity to human colon cancer HCT116 cell line than to a non-malignant one. A number of lead compounds emerged from this study. In particular, **2g**, **2j**, **2m** and **2n** are promising cytotoxins having IC_{50} values in very low micromolar range (0.03-0.04 μM) and very high tumour selective cytotoxicity (SI>300-fold). These compounds displayed >100-fold higher cytotoxic potencies than the reference drug 5-FU. Among these four molecules, **2j**, **2m** and **2n**

satisfy druglike properties favourably. The mode of action studies reveal that the potencies of the cytotoxins in series **2** includes the lowering of the MMP and the generation of ROS. In the future these lead molecules will be evaluated against a wide range of tumours and non-malignant cells to determine which types of malignancies are the most sensitive to these dichloroamides and also further in vivo evaluations will be pursued to find the most promising preclinical candidates for treating colon cancers. Further analog development needs to be pursued vigorously with the aim to find compounds with IC_{50} figures in single digit nanomolar range.

Acknowledgements

The authors thank the Office of the Executive Vice Chancellor for Academic Affairs, Indiana University and the Maunders McNeil Foundation Inc. for financial support for this study.

Conflict of interest

The authors have no competing financial interests to declare.

Supplementary section

Details of the synthesis of the compounds in series **1-4**, quantitative structure-activity relationship determinations, the bioassays and X-ray crystallography are presented on pages S1 to S10 in the supplementary section.

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17. A mixture of **2a** (39 mg), dimethylsulfoxide (10 mL) and 0.1 M phosphate buffer (90 mL) was incubated at 37˚C in a shaker bath. Samples of 10 mL were removed after 0, 24 and 48 h and extracted with chloroform. The organic extract was dried (sodium sulfate) and the solvent was removed to give a residue which was dissolved in deuterochloroform (0.5 mL) and analysed by ^{1}H NMR spectroscopy.
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20. http://www.swissadme.ch. SIB Swiss Institute of Bioinformatics, Molecular Modeling Group, Quartier Sorge, Bâtiment Génopode, CH-1015 Lausanne, Switzerland.

Table 1. Evaluation of **1a, 2a-o, 3** and **4** against HCT116 and CRL1790 cells

Compound	Aryl Substituent	$IC_{50}(\mu M)$[a]		SI[b]
		HCT 116	CRL1790	
1a	H	3.50±0.13	9.45±0.29	2.70
2a	H	0.23±0.054	12.5±0.50	54.4
2b	2-F	0.21±0.059	11.0±0.86	52.4
2c	3-F	0.11±0.033	14.8±0.81	135
2d	4-F	0.05±0.020	14.6±0.67	292
2e	3,4-F_2	0.06±0.023	11.1±0.64	185
2f	4-Cl	0.13±0.014	14.9±0.89	115
2g	3,4-Cl_2	0.04±0.005	12.2±0.56	305
2h	4-OCH_3	0.27±0.033	13.2±0.81	48.9
2i	3,4-$(OCH_3)_2$	0.11±0.033	10.6±0.94	96.4
2j	3,4,5-$(OCH_3)_3$	0.04±0.10	15.1±0.56	378
2k	3,4-OCH_2O	0.32±0.042	21.1±0.98	65.9
2l	4-OH	3.85±0.14	13.8±0.61	3.58
2m	4-CH_3	0.03±0.005	16.2±0.61	540
2n	4-NO_2	0.03±0.006	11.1±0.76	370
2o	4-$N(CH_3)_2$	13.30±2.73	9.12±0.62	0.69
3	H	0.28±0.072	20.8±0.82	74.3
4	$OCOCHCl_2$	0.23±0.067	16.4±0.20	71.3
SDA[c]	- - -	14.8±3.81	12.5±0.94	0.85
5-FU[d]	- - -	4.02±0.883	18.7±0.82	4.65

[a]The IC_{50} value is the concentration of the compound required to inhibit the growth of the cells by 50%.

[b]The letters SI refer to the selectivity index which is the quotient of the IC_{50} values of the compounds towards CRL1790 and HCT 116 cells.

[c]SDA means sodium dichloroacetate.

[d]5-FU refers to 5-fluorouracil.

Table 2. Effect of **2a**, **n**, **o**, **3** on the cell cycle of HCT116 cells.

Compound[a]	Phases of cell cycle[b]				
	Apoptosis	G_0-G_1	S	G_2-M	Ploids
2a	3.6**	44.7*	19.9	29.6	1.8***
2n	3.6*	44.4**	21.4*	27.8	2.2
2o	4.6**	42.9**	20.8*	29.9	1.4**
3	3.5*	45.5	18.3	30.0	2.4
DMSO	2.4	46.8	18.4	29.4	2.5

[a]The concentrations of **2a**, **n**,**o**,**3** used are 0.23, 0.03, 10.96 and 0.28 µM respectively.
[b]The figures are the percentage of the cells in various phases of the cell cycle. Differences between the phases of the cell cycle compared to dimethylsulfoxide are represented as *($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Captions for Schemes and Figures

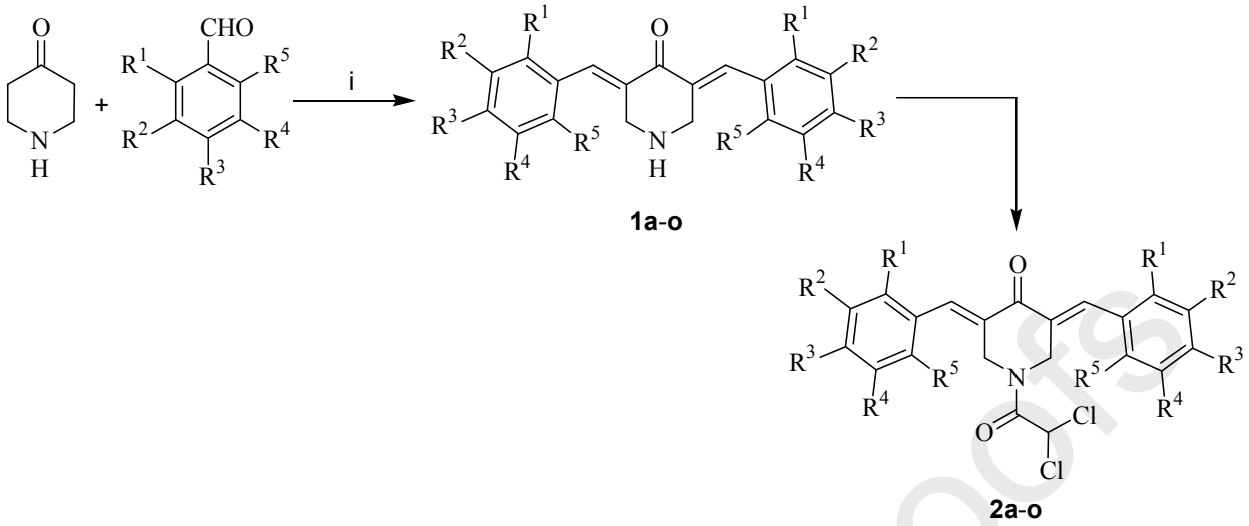
Scheme 1. The synthetic chemical route to the compounds in series **2**. The reagents used are as follows namely i = 10% aqNaOH/ethanol for **1a-k**, **m**, **o**; CH_3COOH/HCl for **1l**, **n**; ii = $Cl_2CHCOCl/N(C_2H_5)_3$/HCl /1,2–dichloroethane, $CHCl_3$. The substituents in the aryl rings in series **2** are represented in table 1.

Scheme 2. The synthetic chemical route to the compounds in series **3** and **4**. The reagents used are as follows: i=10%aqNaOH; ii=$CH_3COCl/N(CH_3)_3$; iii=CH_3COOH/HCl; iv= $(CH_3)_3COCOCl$/tetrahydrofuran, v = $Cl_2CHCOCl/N(C_2H_5)_3$, tetrahydropyran.

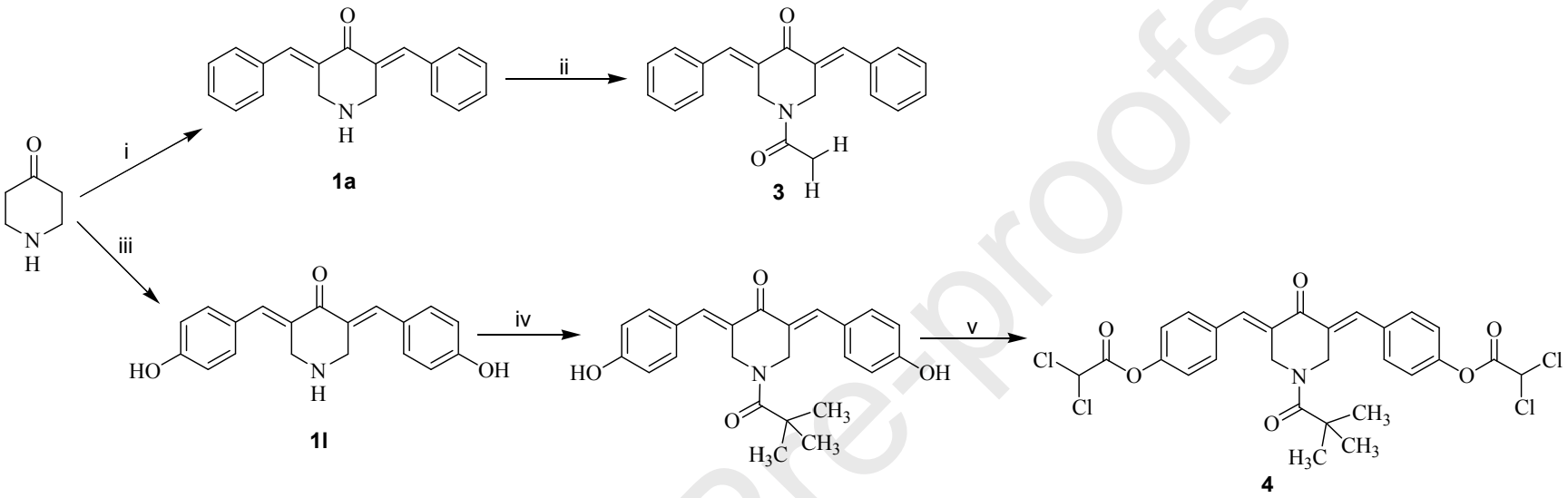
Figure 1. The ORTEP diagram of **2c**.

Figure 2. The effect of **2a**, **n**, **o**, **3** on the mitochondrial membrane potential potential of HCT116 cells.

Figure 3. The effect of **2a**, **n**, **o**, **3** on the production of reactive oxygen species in HCT116 cells.



Scheme 1



Scheme-2

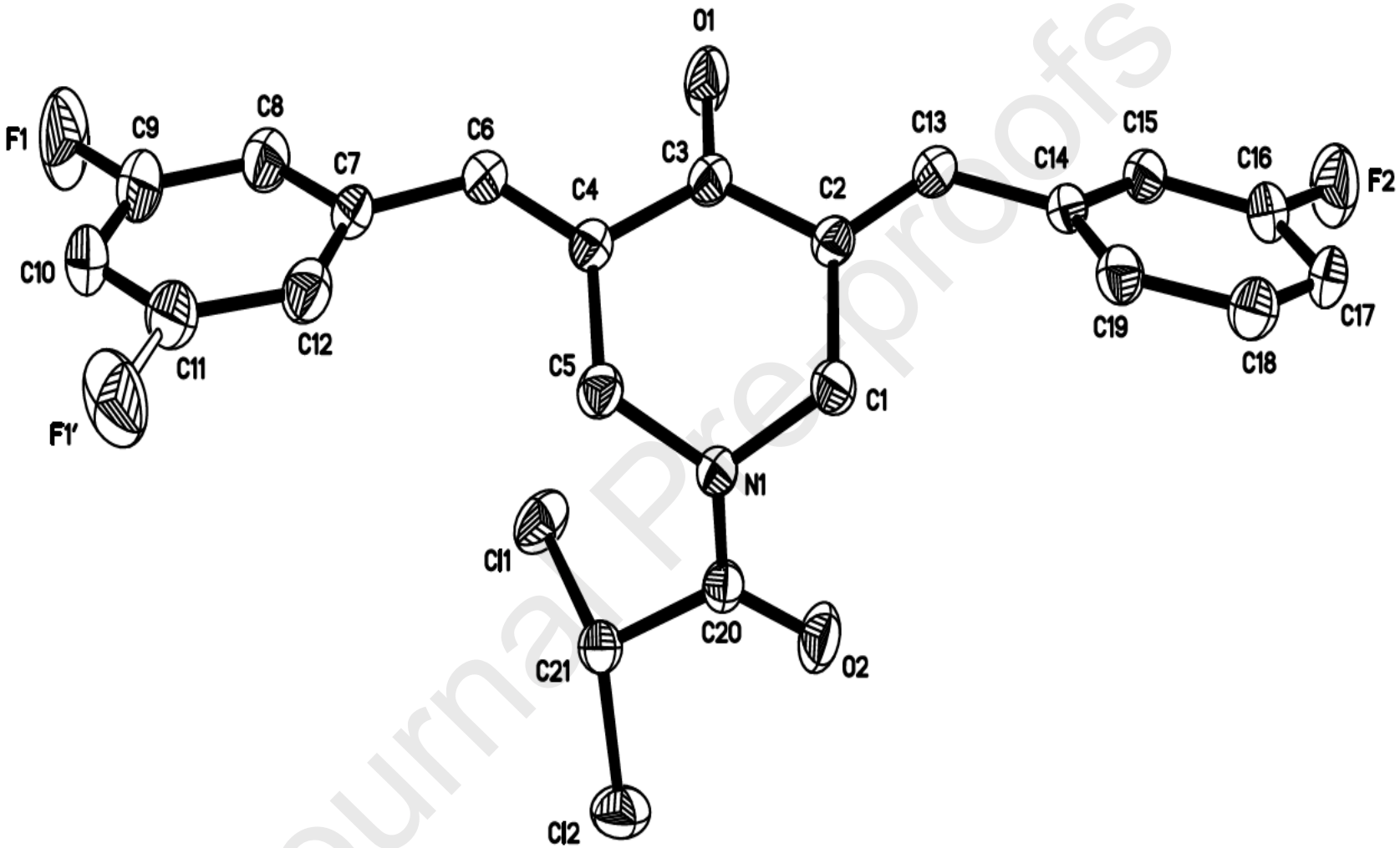


Figure 1

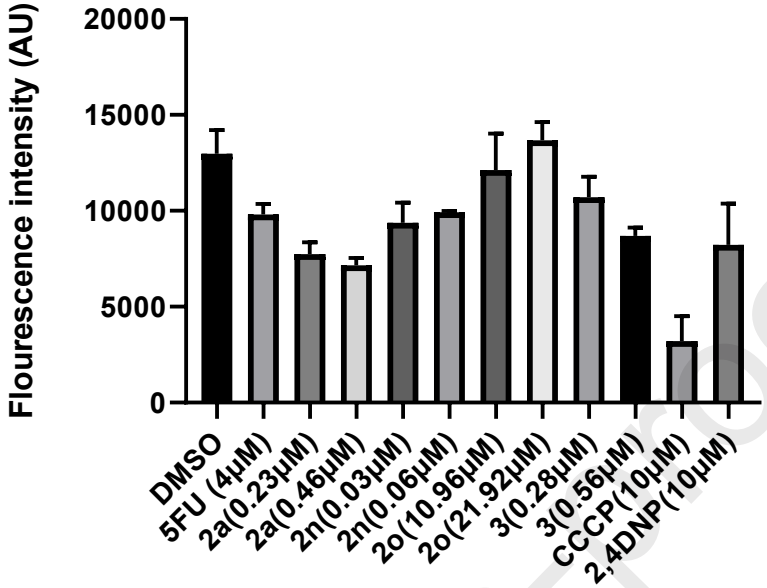


Figure 2

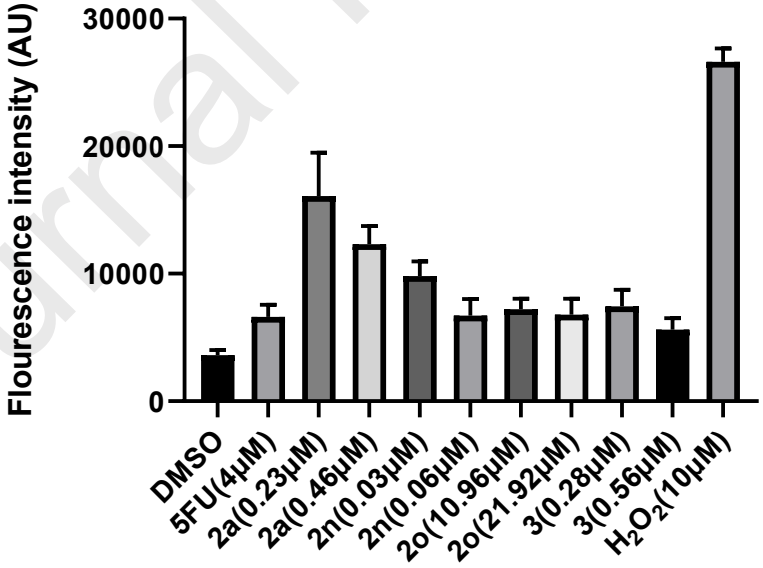
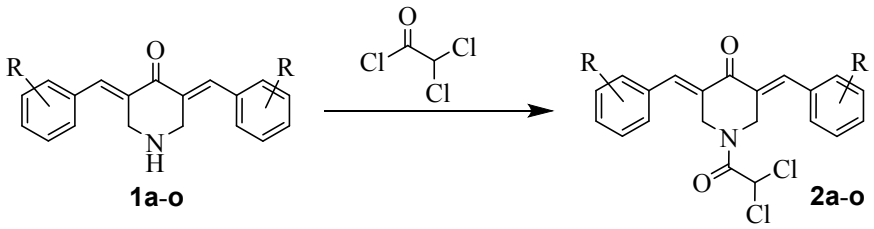


Figure 3

GRAPHICAL ABSTRACT



1a-o **2a-o**

	IC_{50} (HCT116)	IC_{50}(CRL1790)	SI*
2m (R= 4-CH_3)	0.03 0.005	16.2±0.61	540
5-FU	4.02 ± 0.883	18.7±0.82	4.65

*SI: IC_{50} (CRL1790)/IC_{50} (HCT116)

Highlights

- Novel hybrid molecules displaying high potency towards human colon cancer HCT116 cells.
- Most compounds are far less toxic to non-malignant CRL1790 cells leading to high selectivity index values.
- Lead molecules demonstrated >100 times higher potency than a reference drug 5-FU.
- The mode of action of representative compounds includes lowering of the mitochondrial membrane potential and increase in ROS generation.

Declaration of interest statement

The authors have no competing academic and financial interests to declare.