Functionalized Cyclopentenones with Low Electrophilic Character as Anticancer Agents

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In this study were synthesized non-Michael acceptor cyclopentenones (CP) from biomass derivative furfural as anticancer agents. Cyclic enones, both from natural sources and synthetic analogues, have been described as cytotoxic agents. Most of these agents were unsuccessful in becoming valuable therapeutic agents due to toxicity problems derived from unselective critical biomacromolecule alkylation. This may be caused by Michael addition to the enone system. *Ab initio* studies revealed that 2,4-substituted CPs are less prone to Michael additions, and as such were tested three families of those derivatives. We prepare the new CPs from furfural through a tandem furan ring opening/Nazarov electrocyclization and further functionalization. Experimentally the 2,4-substituted CPs exhibited no reactivity towards sulphur nucleophiles, while maintaining cytotoxicity against HT-29, MCF-7, NCI-H460, HCT-116 and MDA-MB 231 cells lines. Moreover, the selected CP are non-toxic against healthy HEK 293T cell lines and present proper calculated drug-like properties.

Estimates from 2012 states colorectal cancer (CRC) as one of the most common cancers in Europe being one of the cancer types that has contributed with more deaths that year, following lung cancer.[1] Fluorouracil (5-FU) is the first-line anti-cancer drug in CRC; but developing of chemoresistance leads to a very poor prognosis.[2] Several anticancer agents are reported every year, being an active research topic.[3–8]

Cyclic enones such as cyclopentenones (CPs) and cyclohexanones have been reported to have cytotoxic activity against several cancer cell lines.[9–15] An example of simple cyclic enones with relevant activities are 2-crotonyloxymethyl-2-cyclopentenone/hexenone/heptenones (COMCs).[16–18] Creighton and coworkers observed that formation of a highly reactive

exocyclic enone is responsible for the activity (Figure 1).[19] This activated species is capable of alkylating critical biomacromolecules such as DNA or proteins.[20] The exocyclic enone is formed in the presence of GSH via thio-Michael addition to the cyclopentenone followed by carboxylate elimination. Moreover, it was shown that the cytotoxicity of COMC correlates to the level of expression of GSTP1-1 when MCF-7[piGST] and MCF-7wt breast tumor cells were incubated with the cytotoxic agents.[10]

This indicates that the exocyclic enones which require GSTP1-1 to be formed are important for cytotoxicity, corroborating the hypothesis that alkylation of critical biomacromolecules is indeed responsible for the activity. However, the presence of this highly reactive Michael acceptor is cause of

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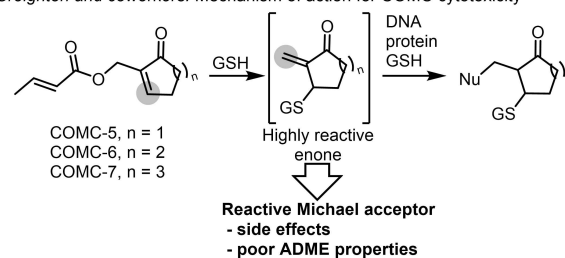Babraham Research Campus, Cambridge, CB22 3AT (UK)

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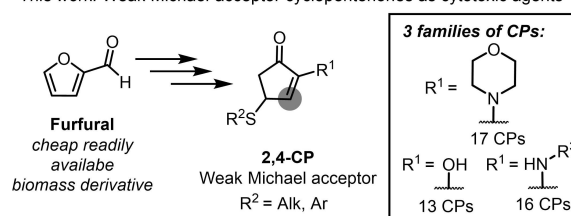Universidade Lusófona, Lisboa, 1749-024 Lisboa (Portugal)

Figure 1. COMCs as anticancer agents described by Creighton and coworkers (top); Preparation of cyclopentenones with poor electrophilic character from furfural (bottom).

concern due to possible promiscuity which can lead to severe side effects.[21,22]

In fact, Michael acceptors are included in the list of compounds that furnished false positives due to high reactivity and lead to the development of filters to exclude the so called Pan Assay Interference Compounds (PAINS) in biological screening.[23]

The synthesis of biologically active products from biomass may allow for cheaper, more sustainable and accessible drug products.[24] Furfural is a cheap furan derivative obtained from non-edible carbohydrates such as xylose. Its conversion to CPs is a topic that has attracted the attention of several research groups.[25]

We have been involved in the preparation of trans-4,5-diaminocyclopentenones from readily available biomass derivative furfural.[26,27] This transformation occurs in a highly selective manner with high yields under mild conditions. In our studies we observed that the addition of a thiol to the enone under basic conditions is followed by consequent elimination of an amine in position 4 to re-establish the enone motif.[28,29] No second addition of the excess thiol to the newly formed enone was observed. Herein we envisioned that the development of CPs with very poor Michael acceptor character could retain the cytotoxic activity through a mechanism of action other than non-specific macromolecule alkylation (Figure 1).

The initial trans-4,5-diamino-cyclopent-2-enones (DCP) can be prepared in water by condensation of furfural with amines promoted by Cu(OTf)$_2$ as previously reported by us and depicted in Scheme 1.[26] This method was chosen not only due to the efficiency of the reaction but also due to potential environmental benefit, by using water as reaction media instead of common organic solvents allowing for the reduction of toxic waste.

DCPs **1**–**5** contain an enone highly prone to Michael addition and as such may undergo conjugation with critical macromolecules leading to in vivo toxicity similar to COMCs.

Ab initio studies using B3LYP/6-31G(d) level of theory were performed to calculate the value of natural bond orbital (NBO) charge distribution from three different cyclopentenones, one belonging to the DCP family (**1**), one to a previously described 2-morpholino-4-thio-cyclopent-2-enones (MCP) family (**18**) and another belonging to also a previously described 2-hydroxy-4-thio-cyclopent-2-enones (HCP) family (**31**). NBO analysis has
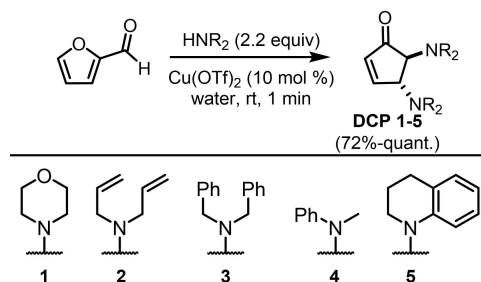
been consistently used to measure electron distribution and therefore predict both nucleophilicity and electrophilicity.[30,31]

The results are summarized in Figure 2, and as expected the β-C both in MCP and HCP possess more negative charge than the β-C present in DCP. The NBO charge density present in the β-C of **1** is comparable to a common enone Michael acceptor reported in the literature[32] and in the case of HCP **31** is comparable to an enone that does not undergo Michael addition.[33] Moreover, we observed that upon stirring MCP **18** with thiophenol, no second Michael addition was observed and after 24 hours the starting material was recovered.

As such, we opted to test these CP derivatives with decreased electrophilicity, therefore being less prone to undergo Michael addition. Whereas the aforementioned DCPs were obtained following the procedure depicted in Scheme 1 (Cu-(OTf)$_2$ in water), the MCP, HCP and ACP derivatives were synthesized by a previously described route[28] as depicted in Scheme 2 (For more detailed information see SI). Firstly, a condensation of morpholine with furfural followed by addition of a thiol under basic conditions furnishes the desired MCP in one pot.

We highlight that the Michael addition occurs followed by elimination of the morpholine in position 4 to re-establish the enone.[28] Next, we prepared HCP by acid promoted hydrolysis of MCP in a methanol/water mixture at 60 °C.[28] We envision that the hydrolysis to HCP would; 1) enhance solubility in aqueous media; 2) allow for additional H-bond since the free alcohol behaves as H bond donor, potentiating interaction with target containing H-bond acceptor groups; 3) maintain the low electrophilic character of the enone, since the olefin is also an
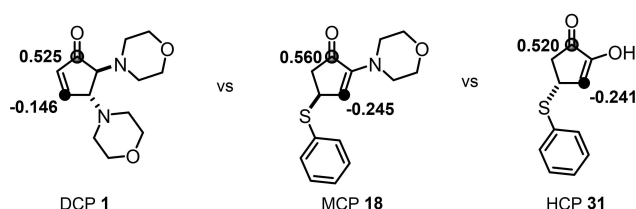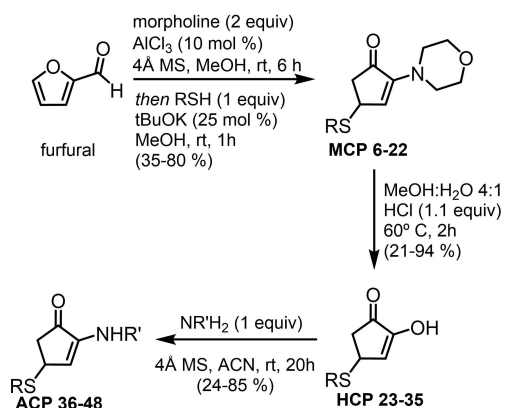
Figure 2. Comparison of the NBO charge distribution on the β-carbon of the enone system between DCP **1**, MCP **18** and HCP **31**.

Scheme 1. Preparation of trans-4,5-diamino-cyclopent-2-enones promoted by Cu(OTf)$_2$ in water.

Scheme 2. Preparation of cyclopentenone derivatives.

enol. Moreover, this modification will give some insights into the importance of the substituent in position 2.

To continue our study on the importance of the substituent in position 2 we prepared 2-amino-4-thio-cyclopent-2-enones (ACP) with secondary amines in the corresponding position.

These amines retain the H-bond donor character of the free alcohol and allows an additional alkyl chain potentiating Van-der-Waals interaction with the still unknown targets.

Moreover, the amines can protonate in the active site to form favorable ionic bonds with anions present in the target. Incorporation of the amines is performed in acetonitrile with 4 Å molecular sieves in order to remove water following the reported procedure.

For the cytotoxic study we used five DCP that were previously reported by us (Scheme 1, **1**–**5**), twelve MCP that were previously reported by our group (Scheme 3, **6**–**17**) and prepared five new MCP containing aryl thiols (Scheme 3, **18**–**22**) under the same conditions in good yields. The HCP family consisted of eight HCP that were previously described by us (Scheme 3, **23**–**30**) and five new HCP (Scheme 3, **31**–**35**) prepared from the hydrolysis of **18**–**22**. These new HCP were isolated by precipitation. Although tautomerism of the HCP to diketone is theoretically possible, the diketone was never observed. Density functional theory studies at the M06-2X/

Def2-TZVPP//B3LYP/6-31G(d) level of theory corroborates this tendency, with a $\Delta G = 5.3$ kcal mol^{-1} favoring the α,β-unsaturated system (See SI). We had previously described the preparation of ACP by condensation of HCP **24** with primary amines (Scheme 3, **36**–**43**). We now extended the procedure by condensation of HCP **31**–**35** with propylamine (Scheme 3, **44**–**48**).

With the different families of compounds in hand firstly, the activity of the new derivatives was evaluated for the antiproliferative activity in the Neutral Red assay at a fixed concentration of 20 µM in HT-29, MCF-7 and NCI-H460 cells (Table 1). Relevant compounds were also tested in HCT-116 and MDA-MB 231 cells and the corresponding IC$_{50}$ was determined.

When we evaluated the activity of DCPs, DCP **1** and **2** were shown to be nontoxic. However, in the case of CP **3**–**5** we observed significantly reduced viability at 20 µM (up to 13% in HT-29, 7% in NCI-H460 and 6% viability in MCF-7). These were additionally tested in HCT-116 and MDA-MB 231 cells and while they retain their activity in HCT-116 cells, a significant decrease in activity was observed in MDA-MB 231 cells. Unlike the MCF-7 cells, the MDA-MB 231 are triple negative breast cancer cells, lacking the estrogen receptors (ER). This result indicates a high dependence of the cytotoxicity on the ER, however it is unclear if the decreased activity is related to decreased uptake of the molecule.

No activity was observed in MCP containing thioalkyl substituents (**6**–**17**) in any of the studied cancer cell lines. Gratefully we observed decreased viability when the cell lines were incubated with MCP **18**–**22**, which contain a thioaryl substituent. Although there was no decrease of viability below the 50% threshold, was observed a decrease of up to 53% viability in NCI-H460 cell lines for MCP **19**. This compound was selected for the determination of IC$_{50}$ discussed further in the article. The MCP family was also tested in HCT-116 and MDA-MB 231 cells and similarly to the previous compounds the activity in ER negative cells was reduced.

Concerning the HCP family, no decrease in viability was observed in thioalkyl substituents (**23**–**30**). However, in HCP containing thioaryl substituents, we observed a significant decrease in cytotoxicity in HT-29 and MCF-7 cell lines upon
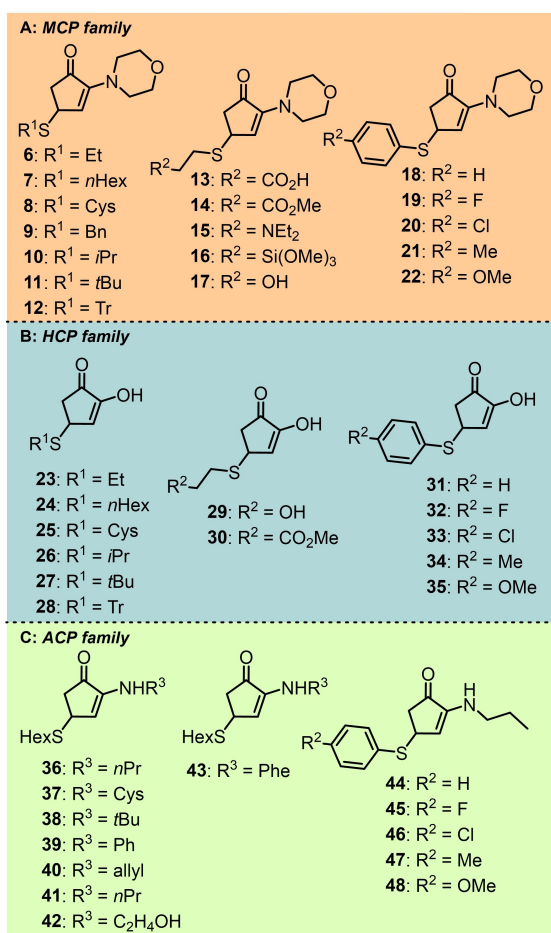
Scheme 3. Cyclopentenones used for the cytotoxic evaluation assays.

Table 1. Heatmap of viability assays at 20 µM CPs on different cell lines.[a]

CP	Viability [%] HT-29	NCI-H460	MCF-7	HCT-116	MDA-MB 231	HEK 293	CP	Viability [%] HT-29	NCI-H460	MCF-7	HCT-116	MDA-MB 231	HEK 293
1	100	100	88	N.D.	N.D.	N.D.	31	90	94	92	100	100	90
2	100	100	100	N.D.	N.D.	N.D.	32	82	85	100	55	54	88
3	28	27	21	34	78	92	33	100	55	76	58	59	100
4	13	7	6	61	85	72	34	67	100	100	100	100	75
5	13	8	6	47	94	90	35	99	96	100	100	100	100
6-17	100	100	100	N.D.	N.D.	N.D.	36-43	100	100	100	100	100	N.D.
18	80	75	60	100	100	100	44	100	76	92	N.D.	N.D.	N.D.
19	62	54	64	100	100	100	45	87	83	79	N.D.	N.D.	N.D.
20	100	65	60	100	100	100	46	100	88	80	N.D.	N.D.	100
21	87	79	84	100	100	98	47	100	81	86	N.D.	N.D.	N.D.
22	86	74	69	100	100	92	48	100	85	86	N.D.	N.D.	N.D.
23-30	100	100	100	N.D.	N.D.	N.D.	Dox	27	18	36	35	54	N.D.

[a] Viability color code: 100% ▭ 0%

replacement of the morpholine with OH in HCP **31**–**35**. Only HCP **33** retained its activity in NCI-H460 cells, being selected for determination of IC$_{50}$. However, when tested in HCT-116 and MDA-MB 231 cells the HCP exhibited increased activity. This is in clear contrast with previous families where the activity was decreased in the ER negative cells. The increased activity suggests that the OH may be involved in important H-bond interaction in the molecular target or even a different mechanism of action in comparison with DCP and MCPs. As such, we envisioned that the incorporation of secondary amines that retain the ability for H-bond interaction in ACPs **44**–**51** would enhance cytotoxic activity. However much to our surprise ACP activities were significantly reduced.

To evaluate selectivity in cancer cell lines, the activity of the most promising compounds was evaluated for the anti-proliferative activity in the MTT assay at a fixed concentration of 20 μM in healthy Human Embryonic Kidney 293 (HEK 293T) cell lines. We observed that while CPs **3**–**5** were most active, they remain slightly cytotoxic in HEK 293T cells. The same was observed for HCP **32**, which exhibited significant activity in HCT-116 and MDA-MB 231 cells. In contrast, HCP **33** shows no toxicity in healthy cell lines, while showing significant activity in the tumorous cell lines.

Overall, DCP containing Michael acceptor exhibit high cytotoxicity. However they may be prone to promiscuous activity due to unspecific 1,4-addition with other biological thiols. Concerning the non-electrophilic CP, aromatic thiol substituents with electron-withdrawing groups show increased activity when compared with other thiols. The morpholine substituent show increased activity in MCF-7 cell lines when compared with the hydrolyzed analogs, which were in turn more active in ER-negative MDA-MB 231 cell lines.

The electronic effect of the thioaryl substituents was evaluated by Hammett plot in the NCI-H460 cell line. When comparing the results of CP **31**–**35** we observe a strong correlation between activity and electronegativity of the aromatic substituent of the thiol. When traced the plot of viability at 20 μM vs Hammett σ$_{para}$ coefficient of the corresponding substituents was linear with a ϱ value of −1.16 for NCI-H60 cells (Figure 3). This indicates that electron-withdrawing groups indeed favor the cytotoxic activity of the CPs. However, the same was not observed for the remaining cell lines.

Prompted by these results, we measured the IC$_{50}$ values of the most active compounds **3**, **4** and **5** belonging to the first family of highly electrophilic DCP, **19** belonging to the MCP family and **32** and **33** belonging to the HCP family.

We observed low IC$_{50}$ values in CP **3**–**5** (up to 3.17 μM in HT-29, 2.16 μM in NCI-H460 and 1.31 μM in MCF-7, Table 2, Entries 1–3). However, these compounds show slight toxicity in HEK 293 cell lines (viabilities at 20 μM of 92%, 72% and 90% respectively, Table 1) and are highly electrophilic enones, prone to react with nucleophiles present in critical biomacromolecules. This is shown by the reduced stability in the presence of glutathione (GSH) shown in assays below.

Both MCP **19** and HCP **33** were close to the 50% viability threshold at 20 μM in NCI-H460 and as such their IC$_{50}$ was
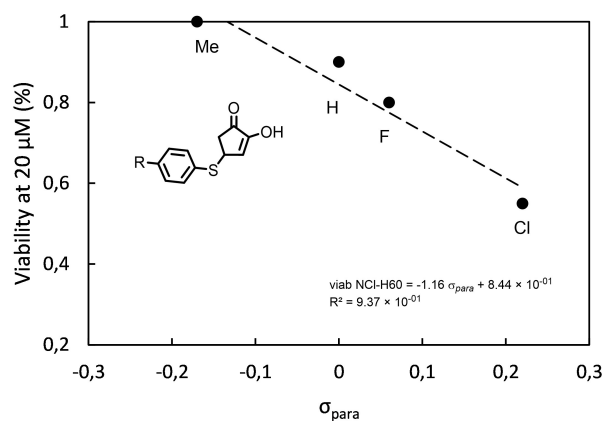

Figure 3. Hammett plot for the biological activity in NCI-H60 cells of different thioaryl substituents on HCP.

$$\text{viab NCI-H60} = -1.16\,\sigma_{para} + 8.44 \times 10^{-01}$$
$$R^2 = 9.37 \times 10^{-01}$$

Table 2. IC$_{50}$ values of the most promising CPs.

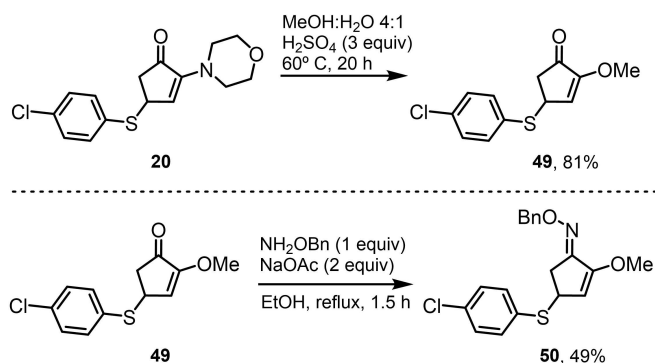| Entry | CP | IC$_{50}$ [μM][a] | | | | |
		HT-29	NCI-H460	MCF-7	HCT-116	MDA-MB 231
1	**3**	3.3±0.6	2.2±0.9	1.8±0.4	–	–
2	**4**	7.6±2.5	5.6±1.0	1.3±0.7	–	–
3	**5**	3.2±0.7	2.4±0.3	2.0±0.4	–	–
4	**19**	–	24.9±2.2	–	–	–
5	**32**	–	–	–	21.9±2.4	17.1±3.6
6	**33**	–	28.7±1.8	–	16.9±1.4	18.0±1.4

[a] Data are the mean ± SD of at least three independent experiments.

determined. Despite higher IC$_{50}$ (24.96 μM and 28.72 μM respectively) than the DCP compounds, these new families were not prone to Michael addition by endogenous thiols and the compounds were not cytotoxic in HEK 293 cells, thus showing some selectivity towards cancer cells.

Additionally, both HCP **32** and **33** exhibited significant activity in HCT-116 and MDA-MB 231 cells and as such their IC$_{50}$ was determined. Whereas **32** had 21.86 μM and 17.07 μM IC$_{50}$ in the respective cell lines, it was slightly toxic in healthy cell lines. However, **33** presented IC$_{50}$ of 16.94 μM and 18.00 μM in the respective cell line without toxicity in the tested concentration.

Knowing that arylthiols present better activity, in particular 4-chloroaryl, and that both the free alcohol and a tertiary amine in position 2 favor the cytotoxic activity in HT-29 cell lines, we decided to explore further the structure-activity relationship of these CPs. At first glance, it appears that H-bond donors are important for the activity. As such we prepared **49**, the methylated derivative of **33**, to prevent H bond interaction. The methylated compound was prepared by methanolysis of **20** promoted by sulfuric acid under long reaction times (Scheme 4, top).

The oxime derivative **50** was also prepared from **49** by condensation with O-benzyl-hydroxylamine promoted by sodium acetate (Scheme 4, bottom). This allows us to study the importance of the ketone on the biological activity. We made attempts at preparing the oxime directly from **33** but were unsuccessful.

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Scheme 4. Cyclopentenone derivatives of **20** prepared for the SAR study. Methanolysis of **20** (top) and oxime formation from **50** (bottom).
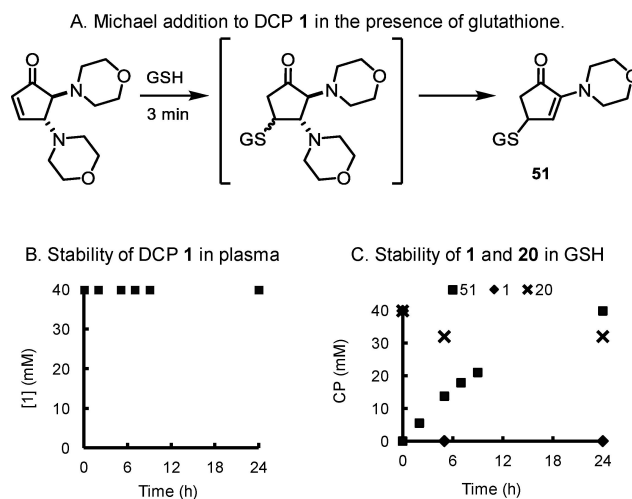

Figure 4. A. Michael addition to DCP **1** in the presence of glutathione. B. Stability of DCP **1** in plasma; C. Incubation of DCP **1**, and **20** with glutathione. Formation of product **51** was measured while studying the stability of DCP **1**. Initial concentration for the stability assays is 40 mM.

With the compounds in hands, we evaluated the activity of the new derivatives of **20** for the anti-proliferative activity at a fixed concentration of 20 μM in HT-29, MCF-7 and NCI-H460 cells (Table 3). A slight increase in activity in HT-29 and MCF-7 cell lines was observed in compounds bearing a methylated substituent in position 2, compared to the free hydroxyl group (Table 3, **33** vs **49**). However a significant decrease in activity was observed in NCI-H460 cells. Such difference might suggest H-bond interactions in this position to be important to the activity. Masking the enone by the formation of the corresponding oxime ether, we observed an even further decrease in activity (Table 3, **50** vs **49**). This result highlights the importance of the ketone in activity.

With the activity of the compounds evaluated, we studied the stability of DCP **1** both in human plasma and in a glutathione solution. By evaluating the stability in presence of GSH we measure the promiscuity of the enone, which is one of the main drawbacks to enones in medicinal chemistry applications.

Incubation of DCP **1** in human plasma shows no degradation by quantitative ^{1}H-NMR after 24 h (Figure 4, B). However, in the presence of GSH we observed complete conversion of **1** by ^{1}H-NMR in 3 minutes (Figure 4, C). The absence of an olefinic proton after 3 minutes, followed by a reappearance of a new olefinic proton over time is consistent with a fast-initial formation of a trisubstituted cyclopentanone intermediate, followed by slow elimination to product **51** (Figure 4A and C).

Incubation of CP **20**, selected as model for non-electrophilic CP, in glutathione was followed by quantitative ^{1}H NMR. Indeed, this CP in comparison with **1** was not susceptible to Michael addition, with no formation of a glutathione adduct even after 24 hours.

Even though there is no Michael addition, we observed a slight decrease in the starting CP concentration over time (Figure 4C). The substituent in position 2 undergoes amine exchange with the primary amine in glutathione, through a mechanism similar to the formation of ACP **36**–**48** from HCP. This effect is more pronounced in HCP **33** which is more susceptible to the amine exchange (For more information see SI).

Finally, the drug-like properties of the new CP were calculated (Table 4). The active compounds present good drug-like properties, with low molecular weights, calculated LogP (cLogP) between 2.52 to 4.31, with the exception of DCP **3** where the cLogP is 5.49. Hydrogen bond donors (HBD) between 0–1 and only 2–3 hydrogen bond acceptors (HBA) and TPSA between 23, 55 and 37.3. The calculated properties fit the Lipinski's rule of 5 and also the rules described by Veber et al.[34] < 140 PSA and < 12 rotatable bonds.

Overall, although some of the initial DCP show remarkable anticancer activity, we believe the MCP family to be the most promising. This is due to the high activity aligned with low toxicity in healthy cell lines and better stability profile in the presence of GSH.

In conclusion *ab initio* studies allowed us to select and prepare three different families of cyclopentenones from furfural as a cheap and easily available biomass derivative. The

Table 3. Viability assays of derivatives of CP **20** on different cell lines.

Compound	Viability [%] (20 μM)[a]		
	HT-29	NCI-H460	MCF-7
20	100	64 ± 1.1	60 ± 15.7
33	100	55 ± 5.8	76 ± 4.0
49	80 ± 11.0	82 ± 6.2	60 ± 18
50	94 ± 13.4	100	96 ± 10.6
[a] Data are the mean ± SD of at least three independent experiments.			

Table 4. Calculated properties of the most promising CPs.

Entry	CP	cLogP	HBD	HBA	MW	TPSA	FR
1	**3**	5.49	0	3	472.6	23.55	10
2	**4**	3.29	0	3	292.4	23.55	4
3	**5**	4.31	0	3	344.5	23.55	2
4	**33**	2.52	1	2	240.7	37.3	2

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cyclopentenones show promising results as anticancer agents against HT-29, MCF-7 and NCI-H460 cells lines. Additionally, while DCP and MCP lose their activity in ER-negative breast cancer cells, the HCP family exhibits more activity in these cell lines. Unlike the commonly used enone cytotoxic agents, the new cyclopentenones lack the strong electrophilic character that leads to noxious side effects *via* unselective alkylation of critical macro biomolecules. Moreover, we observed that active cyclopentenones do not undergo Michael addition in the presence of glutathione. Computational studies indicate good drug-like properties according to the Lipinski rules of 5.

Experimental Section

General remarks

All solvents were distilled prior to use. Furfural was distilled and stored at 4 °C. Unless otherwise stated, all reagents were used as received from commercial suppliers. Reaction progress was monitored by thin-layer chromatography (TLC) performed on aluminium plates coated with silica gel F254 with 0.2 mm thickness. ^{1}H and ^{13}C NMR spectra were acquired on Bruker MX300 spectrometer.

General Procedure A for the Preparation of trans-4,5-Diamino-cyclopent-2-enones (1–5)

To a solution of $Cu(OTf)_2$ (40 mg, 10 mol%) in water (1 mL) were added amine (2.29 mmol, 2.2 equiv) and furfural (100 mg, 1.04 mmol). The reaction was allowed to stir vigorously at room temperature for 1 min. Then, the reaction mixture was diluted with water (9 mL) and extracted with diethyl ether (3×10 mL). The combined organic phases were dried with $MgSO_4$, and the solvent was evaporated under reduced pressure to yield the pure cyclopent-2-enones.

General Procedure B for the Preparation of 2-morpholino-4-thio cyclopentenones from 2-furaldehyde (6–22)

To a solution of 2-furaldehyde (0.41 mL, 5 mmol) in dry MeOH (20 mL, 0.25 M) was added morpholine (0.86 mL, 2.2 equiv, 10 mmol), $AlCl_3$ (67 mg, 0.1 equiv, 0.5 mmol) and 4 Å molecular sieves (0.2 g/mmol of 2-furaldehyde). The mixture was stirred for six hours at room temperature under nitrogen atmosphere. Then, was added 1 equivalent of the corresponding substituted thiol and KO^{t}Bu (140 mg, 0.25 equiv, 1.25 mmol). The mixture was stirred at room temperature under nitrogen atmosphere for 1–2 h. Afterwards, the crude was filtered through a short plug of celite and the filter cake was washed with DCM (60 mL). To the filtrate was added AcOH/NaOAc buffer solution at pH 5 (20 mL) and brine (10 mL). The organic layer was separated, and the aqueous layer was further extracted with DCM (2×20 mL), and the combined organic layers were dried with anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to afford the pure products.

4-((4-fluorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (19)

The titled compound was prepared using 4-fluorobenzenethiol (640 mg) according to general procedure **B**. The product was purified by flash chromatography on silica using EtOAc/n-hexane (1:9 v/v) and was isolated as a light brown solid in 43% (631 mg). m.p. 88–89 °C.

^{1}**H NMR (300 MHz, $CDCl_3$) δ** 7.48–7.35 (2H, m), 7.09–6.97 (2H, m), 6.15 (1H, d, J=3.1 Hz), 4.21 (1H, ddd, J=6.3, 3.1, 1.8 Hz), 3.75 (4H, t, J=4.8 Hz), 3.10 (4H, overlapped t, J=4.8 Hz), 2.87 (1H, dd, J=19.2, 6.3 Hz), 2.46 (1H, dd, J=19.2, 1.8 Hz) ^{13}**C NMR (75 MHz, $CDCl_3$) δ** 202.0, 150.9, 136.1, 136.0, 130.6, 128.1, 128.1, 116.5, 116.2, 66.5, 47.9, 43.6, 43.5 **HRMS (ESI-MS)** *m/z* calcd for $C_{15}H_{17}FNO_2S^+$ [M+H]$^+$ 294.0959, found 294.0959.

4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (20)

The titled compound was prepared using 4-chlorobenzenethiol (723 mg) according to general procedure **B**. The product was purified by flash chromatography on silica using EtOAc/n-hexane (1:9 v/v) and was isolated as a dark yellow solid in 54% (836 mg). m.p. 88–90 °C.

^{1}**H NMR (300 MHz, $CDCl_3$) δ** 7.31 −7.19 (4H, m), 6.08 (1H, d, J= 3.2 Hz), 4.21 (1H, ddd, J=6.3, 3.2, 1.8 Hz), 3.68 (4H, t, J= 4,8 Hz), 3.05 (4H, t, J=4.9 Hz), 2.83 (1H, dd, J=19.2, 6.3 Hz), 2.40 (1H, dd, J=19.2, 1.8 Hz) ^{13}**C NMR (75 MHz, $CDCl_3$) δ** 201.8, 150.9, 134.1, 134.0, 132.1, 130.0, 129.4, 66.5, 47.9, 43.5 **HRMS (ESI-MS)** *m/z* calcd for $C_{15}H_{17}ClNO_2S^+$ [M+H]$^+$ 310.0663, found 310.0660.

2-morpholino-4-(p-tolylthio)cyclopent-2-en-1-one (21)

The titled compound was prepared using 4-methylbenzenethiol (621 mg) according to general procedure **B**. The product was purified by flash chromatography on silica using EtOAc/n-hexane (1:9 v/v) and was isolated as a dark brown solid in 35% (506 mg). m.p. 87–88 °C.

^{1}**H NMR (300 MHz, $CDCl_3$) δ** 7.21 (2H, d, J=8.0 Hz), 7.02 (2H, d, J= 8.0 Hz), 2.23 (3H, s), 6.08 (1H, d, J=3.2 Hz), 4.12 (1H, ddd, J=6.3, 3.2, 1.8 Hz), 3.03–2.96 (4H, t, J=4,8 Hz), 3.67–3.57 (4H, t, J=4,8 Hz), 2.76 (1H, dd, J=19.2, 6.2 Hz), 2.37 (1H, dd, J=19.2, 1.8 Hz) ^{13}**C NMR (75 MHz, $CDCl_3$) δ** 202.2, 150.7, 138.3, 133.6, 131.3, 129.9, 129.4, 66.49, 47.9,, 43.5, 43.1, 21.3 **HRMS (ESI-MS)** *m/z* calcd for $C_{16}H_{20}NO_2S^+$ [M+H]$^+$ 290.1209, found 290.1208.

4-((4-methoxyphenyl)thio)-2-morpholinocyclopent-2-en-1-one (22)

The titled compound was prepared using 4-methoxybenzenethiol (701 mg) according to general procedure **B**. The product was purified by flash chromatography on silica using EtOAc/n-hexane (1:9 v/v) and was isolated as a brown solid in 47% (718 mg). m.p. 105–107 °C.

^{1}**H NMR (300 MHz, $CDCl_3$) δ** 7.41–7.32 (2H, m), 6.88–6.80 (2H, m), 6.17 (1H, d, J=3.2 Hz), 4.13 (1H, ddd, J=6.3, 3.1, 1.7 Hz), 3.78 (3H, s), 3.72 (4H, t, J=3.8 Hz), 3.07 (4H, t, J=3.8 Hz), 2.81 (1H, dd, J= 19.2, 6.3 Hz), 2.44 (1H, dd, J=19.2, 1.7 Hz) ^{13}**C NMR (75 MHz, $CDCl_3$) δ** 202.3, 160.2, 150.7, 136.5, 131.7, 122.9, 114.7, 66.5, 55.5, 48.0, 43.8, 43.3 **HRMS (ESI-MS)** *m/z* calcd for $C_{16}H_{20}NO_3S^+$ [M+H]$^+$ 306.1158, found 306.11520.

General Procedure C for the Preparation of 2-hydroxyl cyclopentenones (23-35)

To a solution of the selected 2-morpholino-4-thio-cyclopentenone (1 mmol) in a mixture of MeOH/water (0.2 M, 4:1 v/v) was added HCl 37% (0.094 ml, 1.1 equiv, 1.1 mmol). The mixture was stirred at

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60 °C for 2 hours. Afterwards, water (4 mL) and DCM (12 mL) were added, and layers were separated. Aqueous layer was further extracted with DCM (12 mL). Combined organic layers were dried (MgSO$_4$), filtered and concentrated under reduced pressure to yield a yellow low melting point solid or an orange oil which was purified by flash chromatography on silica.

4-((4-fluorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (32)

The titled compound was prepared using enone **19** (293 mg) according to general procedure **C**. The product was purified by flash chromatography on silica using DCM/MeOH (20:1 v/v) and was isolated as a brown oil in 27% (60 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.47–7.37 (2H, m), 7.08–6.98 (2H, m), 6.46 (1H, d, J = 3.0 Hz), 4.22 (1H, ddd, J = 6.0, 3.1, 1.5 Hz), 2.88 (1H, dd, J = 19.6, 6.0 Hz), 2.44 (1H, dd, J = 19.6, 1.5 Hz) ^{13}C NMR (75 MHz, CDCl$_3$) δ 200.7, 153.1, 136.4, 136.3, 128.4, 116.4, 116.2, 42.3, 40.1 HRMS (ESI-MS) m/z calcd for C$_{11}$H$_8$FO$_2$S$^-$ [M-H]$^-$ 223.0235, found 223.0233.

4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (33)

The titled compound was prepared using enone **20** (310 mg) according to general procedure **C**. The product was isolated by precipitation as a brown solid in 25% (60 mg). m.p. 135 °C–138 °C

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.39–7.23 (5H, m), 6.41 (1H, d, J = 3.0 Hz), 4.21 (1H, ddd, J = 6.0, 3.1, 1.5 Hz), 2.85 (1H, dd, J = 19.6, 6.0 Hz), 2.38 (1H, dd, J = 19.6, 1.5 Hz) ^{13}C NMR (75 MHz, CDCl$_3$) δ 201.0, 153.7, 134,7, 131.2, 129.5, 128.7, 42.0, 40.5, 31.1 HRMS (ESI-MS) m/z calcd for C$_{11}$H$_{10}$ClO$_2$S$^+$ [M+H]$^+$ 241.0085, found 241.00789.

2-hydroxy-4-(p-tolylthio)cyclopent-2-en-1-one (34)

The titled compound was prepared using enone **21** (289 mg) according to general procedure **C**. The product was isolated by precipitation as a brown solid in 24% (53 mg). m.p. 62 °C - 64 °C.

^{1}H NMR (300 MHz, CDCl3) δ 7.38–7.29 (2H, m), 7.18–7.07 (2H, m), 6.5 (1H, d, J = 3.0 Hz), 4.23 (1H, ddd, J = 5.9, 3.0, 1.5 Hz), 2.88 (1H, dd, J = 19.6, 5.9 Hz), 2.46 (1H, dd, J = 19.7, 1.5 Hz), 2.34 (3H, s) ^{13}C NMR (75 MHz, CDCl3) δ 201.5, 153.4, 138.7, 134.1, 130.1, 129.6, 128.7, 42.12, 40.5, 21.3 HRMS (ESI-MS) m/z calcd for C$_{12}$H$_{13}$O$_2$S$^+$ [M+H]$^+$ 221.0631, found 221.06252.

2-hydroxy-4-((4-methoxyphenyl)thio)cyclopent-2-en-1-one (35)

The titled compound was prepared using enone **22** (305 mg) according to general procedure **C**. The product was isolated by precipitation as a brown oil in 21% (50 mg) as a 9:1 mixture of product and SM.

^{1}H NMR (300 MHz, CDCl3) δ 7.64–7.30 (4H, m), 6.96–6.74 (4H, m), 6.47 (1H, d, J = 3.1 Hz), 4.14 (1H, ddd, J = 6.1, 3.1, 1.5 Hz), 3.80 (s, 3H), 2.84 (1H, dd, J = 19.6, 5.9 Hz), 2.44 (1H, dd, J = 19.5, 1.5 Hz), ^{13}C NMR (75 MHz, CDCl3) δ 201.4, 160.4, 153.2, 136.9, 129.6, 122.1, 114.8, 55.5, 42.6, 40.2, HRMS (ESI-MS) m/z calcd for C$_{12}$H$_{13}$O$_3$S$^+$ [M+H]$^+$ 237.0580, found 237.05754.

General Procedure D for the Preparation of 2-amino-4-thio cyclopentenones (44–48)

To a solution of the selected hydrolysed cyclopentenone (0.5 mmol) in dry MeCN (0.25 M) was added n-propylamine (1 eq.) and 4 Å molecular sieves (0.2 g/mmol of diketone). The mixture was stirred at room temperature for 3 hours. Afterwards, the reaction mixture was filtered through a short plug of celite, and the filter cake was washed with DCM. The filtrate was concentrated under reduced pressure to yield a dark brown oil which was purified by flash chromatography on silica.

4-(phenylthio)-2-(propylamino)cyclopent-2-en-1-one (44)

The titled compound was prepared using enone **31** (120 mg) according to general procedure D. The product was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a light brown oil in 82% yield (116 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.44–7.34 (2H, m), 7.33–7.19 (3H, m), 5.77 (1H, d, J = 3.1 Hz), 4.41–4.34 (1H, m), 2.99–2.81 (3H, m), 2.48 (1H, dd, J = 19.4, 1.5 Hz), 1.60–1.41 (2H, m), 0.91 (3H, t, J = 7.4 Hz) ^{13}C NMR (75 MHz, CDCl$_3$) δ 201.5, 146.6, 134.5, 131.8 (2 C), 128.9 (2 C), 127.2, 117.8, 45.8, 43.9, 42.4, 22.0, 11.5 HRMS (ESI-MS) m/z calcd for C$_{14}$H$_{18}$NOS$^+$ [M+H]$^+$ 248.1104, found 248.10952.

4-((4-fluorophenyl)thio)-2-(propylamino)cyclopent-2-en-1-one (45)

The titled compound was prepared using enone **32** (100 mg) according to general procedure D. The product was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a light brown oil in 71% yield (83 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.47–7.35 (2H, m), 7.04–6.91 (2H, m), 5.73 (1H, d, J = 3.0 Hz), 4.34–4.23 (1H, m), 2.99–2.76 (3H, m), 2.44 (1H, dd, J = 19.4, 1.5 Hz), 1.67–1.42 (2H, m), 0.91 (3H, t, J = 7.5 Hz) ^{13}C NMR (75 MHz, CDCl$_3$) δ 201.3, 164.1, 160.8, 146.7, 135.3, 128.8, 117.7, 116.0, 115.7, 44.7, 42.02, 21.9, 11.3 HRMS (ESI-MS) m/z calcd for C$_{14}$H$_{17}$FNOS$^+$ [M+H]$^+$ 266.1009, found 266.0998.

4-((4-chlorophenyl)thio)-2-(propylamino)cyclopent-2-en-1-one (46)

The titled compound was prepared using enone **33** (100 mg) and n-propylamine (0.041 mL) according to general procedure **D**. The product was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a light brown oil in 80% yield (93 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.37–7.09 (4H, m), 5.67 (1H, d, J = 3.2 Hz), 4.28 (1H, ddd, J = 6.0, 3.2, 1.6 Hz), 2.93–2.75 (3H, m), 2.39 (1H, dd, J = 19.4, 1.6 Hz), 1.56 - 1.42 (2H, m), 0.87 (3H, t, J = 7.4 Hz) ^{13}C NMR (75 MHz, CDCl$_3$) δ 201.6, 147.0, 133.7, 133.6 (2 C), 132.9, 129.2 (2 C), 117.6, 46.0, 44.4, 42.4, 22.2, 11.6 HRMS (ESI-MS) m/z calcd for C$_{14}$H$_{17}$ClNOS$^+$ [M+H]$^+$ 282.07140, found 282.07037.

2-(propylamino)-4-(p-tolylthio)cyclopent-2-en-1-one (47)

The titled compound was prepared using enone **34** (100 mg) and n-propylamine (0.041 mL) according to general procedure **D**. The product was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a light brown oil in 66% yield (78 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.38–7.24 (2H, m), 7.15–7.06 (2H, m), 5.76 (1H, d, J = 3.2 Hz), 4.30 (1H, ddd, J = 6.0, 3.1, 1.5 Hz), 3.00–2.76 (3H, m), 2.46 (1H, dd, J = 19.5, 1.5 Hz), 1.64–1.45 (m, 2H), 0.91 (3H, t,

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$J=7.4$ Hz) **^{13}C NMR (75 MHz, CDCl$_3$) δ** 201.8, 146.6, 137.6, 132.9 (2 C), 130.3, 129.7 (2 C), 118.43, 45.8, 44.4, 42.2, 22.0, 21.1, 11.5 **HRMS (ESI-MS)** m/z calcd for C$_{15}$H$_{20}$NOS$^+$ [M+H]$^+$ 262.1260, found 262.12502.

4-((4-methoxyphenyl)thio)-2-(propylamino)cyclopent-2-en-1-one (48)

The titled compound was prepared using enone **35** (100 mg) and n-propylamine (0.041 mL) according to general procedure **D**. The product was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a light brown oil in 78% yield (91 mg).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.36 (2H, d, J=8.9 Hz), 6.82 (2H, d, J=8.8 Hz), 5.74 (1H, d, J=3.1 Hz), 4.20 (1H, ddd, J=5.9, 3.1, 1.5 Hz), 2.99–2.72 (3H, m), 2.43 (1H, dd, J=19.5, 1.6 Hz), 1.64–1.45 (2H, m), 0.91 (3H, t, J=7.4 Hz) **^{13}C NMR (75 MHz, CDCl$_3$) δ** 202.1, 159.9, 146.6, 136.1 (2 C), 123.7, 119.1, 114.6 (2 C), 55.4, 46.0, 45.22, 42.1, 22.2, 11.6 **HRMS (ESI-MS)** m/z calcd for C$_{15}$H$_{20}$NO2S$^+$ [M+H]$^+$ 278.1209, found 278.10952.

Procedure for the preparation of 4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one (49)

To a solution of the selected enone **20** (1.2 g, 3.87 mmol) in a mixture of MeOH/water (0.25 M 4:1 v/v) was added H$_2$SO$_4$ (0.62 mL, 3 equiv, 11.6 mmol). The mixture was stirred at 60°C for 20 hours. Afterwards, water (30 mL) and DCM (20 mL) were added and the aqueous layer was further extracted with DCM (30 mL). The combined organic layers were dried (MgSO$_4$), filtered and concentrated under reduced pressure. The compound was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a brown solid in 81% (799 mg). m.p. 62°C–64°C.

^{1}H NMR (300 MHz, CDCl$_3$): 7.32–7.16 (4H, m), 6.21 (1H, d, J=2.9 Hz), 4.22 (1H, ddd, J=6.6, 3.1, 1.7 Hz), 3.64 (3H, s, 3H), 2.83 (1H, dd, J=19.5, 6.2 Hz, 1H), 2.37 (1H, dd, J=19.5, 1.5 Hz), **^{13}C NMR (75 MHz, CDCl$_3$):** 199.0, 158.2, 134.2, 134.1, 131.5, 129.3, 125.9, 57.5, 41.6, 41.4 **HRMS (ESI-MS)** m/z calcd for C$_{12}$H$_{12}$ClO2S$^+$ [M+H]$^+$ 255.0241, found 255.02360.

Procedure for the preparation of (E)-4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one O-benzyl oxime (50)

To a solution of the selected enone **49** (50 mg, 0.196 mmol) in ethanol (0.2 M) was added O-benzylhydroxylamine (0.023 ml, 1 equiv) and NaOAc (2 equiv) The mixture was refluxed at 40°C for 1,5 hours and afterwards washed with water (20 mL) and extracted with DCM (20 mL). The combined organic layers were dried (MgSO$_4$), filtered and concentrated under reduced pressure. The compound was purified by flash chromatography (EtOAc/n-hexane 1:9) and was isolated as a dark brown oil in 49% (34.6 mg).

^{1}H NMR (300 MHz, CDCl$_3$): 7.50–7.07 (9H, m), 5.32 (1H, d, J=2.8 Hz), 5.09 (2H, s), 4.19 (1H, ddd, J=6.8, 2.8, 1.7 Hz), 3.68 (3H, s), 3.08 (1H, dd, J=19.2, 6.8 Hz), 2.71 (1H, dd, J=19.2, 1.7, 0.6 Hz) **^{13}C NMR (75 MHz, CDCl$_3$)** 157.0, 156.2, 137.5, 133.6, 133.2, 133.0, 129.3, 128.5, 128.3, 128.0, 111.1, 76.8, 57.5, 45.5, 34.0 **HRMS (ESI-MS)** m/z calcd for C$_{19}$H$_{19}$ClNO2S$^+$ [M+H]$^+$ 360.0820, found 360.08133.

Biological assays

General remarks

The in vitro anticancer studies were carried out in Human breast cancer cells line (MCF-7 and MDA-MB-231), human colorectal cancer cells line (HT-29 and HCT-116) and Human lung cancer cells line (NCI-H460) by a Neutral Red Viability Assay, which is a common method based on the detection of viable cells via the uptake of one dye Neutral Red (a Eurhodin dye those stains lysosomes in viable cells). Cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic antimycotic solution in 75 cm^2 tissue culture flasks and incubated with a humidified 5% CO$_2$ atmosphere at 37°C.

The in vitro cytotoxicity studies were carried out in human embryonic kidney 293T healthy cells line (HEK 293T) by a MTT assay which is a colorimetric assay used to measure cellular metabolic activity based on the reduction of a yellow tetrazolium salt to purple formazan crystals by metabolically active cells. Cells were cultured in DMEM medium supplemented with 10% FBS and antibiotic antimycotic solution (1%) in 75 cm^2 tissue culture flasks and incubated with a humidified 5% CO2 atmosphere (at 37°C).

Both cancer and healthy cell lines were purchased from the American Type Culture Collection (ATCC). Cell culture RPMI-1640 Medium, Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA solution, Stabilized Antibiotic Antimycotic solution (100x), and Hanks' Balanced Salt solution (HBSS) were purchased from Sigma. Fetal Bovine Serum (FBS) was purchased from VWR and Dimethyl Sulfoxide (DMSO) from Carlo Erba.

Viability assay against cancer cells

MDA-MB-231, HCT-116 and NCI-H460 human cancer cells were seeded in 96-well plates with RPMI-1640 (supplemented with 10% FBS and 1% antibiotic antimycotic solution) at the concentration of 5×104 cell/mL and were incubated for 24 h (in a humidified 5% CO2 atmosphere at 37°C). After that, stock solutions of compounds were prepared in culture medium with DMSO ($\leq$0.5% (v/v)) and then incubated (in triplicate) with the cells for 48 h. After incubation, the plates were treated with a medium containing Neutral Red (50 μg/mL) and incubated again for more 3 h. Then, medium was removed, plates were washed with HBSS and an organic acid solution of 20 mL ethanol + 20 mL H2O + 400 μL glacial acetic acid was added. Finally, absorbances were measured by spectrophotometry at 540 nm and the cells viabilities calculated. The dose to attain 50% viability (IC50) was determined using GraphPad Prism 5

Viability assay against healthy cells

HEK 293T cells were seeded in a 96-well plate at a concentration of 7×10^4 cell/mL (in DMEM with 10% FBS and 1% antibiotic antimycotic solution) and were incubated for 24 h (in a humidified 5% CO2 atmosphere at 37°C). After that, stock solutions of compounds were prepared in culture medium with DMSO ($\leq$0.5% (v/v)) and then incubated (in triplicate) with the cells for 48 h. After incubation, the plates were treated with a medium containing MTT and incubated again for more 3 h. Then, medium was removed, plates were washed with HBSS and DMSO was added. Finally, absorbances were measured by spectrophotometry at 595 nm and the cells viabilities calculated. The dose to attain 50% viability (IC50) was determined using GraphPad Prism 5.

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Stability assays

The stability assays were performed by quantitative 1H NMR spectroscopy using a coaxial capillary containing sodium acetate as internal standard.

Plasma stability assays

The CP (0.02 mmol) was dissolved in a mixture of DMSO-d_6: plasma (0.4 mL : 0.1 mL). A coaxial capillary containing sodium acetate (0.02 mmol) in DMSO-d_6 was added to the NMR tube. ^{1}H NMR was performed every 30 min.

GSH stability assays

The CP (0.02 mmol) was dissolved in a mixture of DMSO-d_6: buffer (0.4 mL : 0.1 mL). GSH (0.05 mmol) was added. A coaxial capillary containing sodium acetate (0.02 mmol) in DMSO-d_6 was added to the NMR tube. ^{1}H NMR was performed every 30 min.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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