University of Szeged
Faculty of Pharmacy
Institute of Pharmacognosy

Preparation of bioactive oxidized hydroxycinnamic acid derivatives

Ph.D. Thesis Summary

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Preparation of bioactive oxidized hydroxycinnamic acid derivatives

Doctoral dissertation theses

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Introduction

Hydroxycinnamic acids (HCAs), as one of the main groups of phenolic acids, are widespread secondary plant metabolites. They are widely recognized as dietary antioxidants, as they are present in fruits, grains, vegetables, spices, nuts, and beverages such as tea, coffee, and wine.

The basic skeleton of HCAs is a phenylpropanoid structure that consists of nine carbon atoms (C6C3). The most common representatives of HCAs are *p*-coumaric, caffeic, ferulic and sinapic acids. They occur in nature in their free forms or esters, and they can form more complex derivatives such as dimers, trimers etc.

Antioxidants have attracted an increasing scientific interest in the past few decades, owing to their various alleged health benefits and their abundance in dietary plants. Antioxidants can scavenge reactive oxygen and nitrogen species (ROS, RNS), and from this interaction, possible bioactive metabolites of the antioxidants may be formed. Biomimetic oxidative chemical reactions can be considered as relevant models of ROS and RNS scavenging, therefore they provide a valuable toolkit to discover such metabolites.

There are several previous studies about biomimetic oxidative transformations of hydroxycinnamic acids. For example, when ester derivatives of cinnamic acids were oxidized with catalytic amount of silver oxide (Ag_2O), dihydrobenzofuran lignans were formed with potent antitumor activities such as antiproliferative activity, antiangiogenic and antitubulin activity.

A simple and quick semi-synthesis of protoapigenone from apigenin with a hypervalent iodine reagent, PIFA was previously reported by our research group. PIFA may also be considered as “biomimetic-like”, as it can oxidize phenolic compounds through single-electron transfer that is an important mechanism of ROS scavenging by small-molecule antioxidants including HCAs.

The metabolites arising from ROS and RNS scavenging by antioxidants may have in some cases stronger bioactivities then their parent compounds.

This PhD work aimed to evaluate free radical scavenging-mediated formation of hydroxycinnamic acid metabolites by using different oxidative chemical approaches, and to find compounds with promising bioactivity.

Objectives

For the Ph.D. work presented in this dissertation, the following objectives were set up:

1. **Preparation of semi-synthetic oxidized hydroxycinnamic acid derivatives.** The formation of HCA metabolites was investigated in the reaction with different kinds of biomimetic or bio-inspired oxdative reagents, e.g.; hypervalent iodine compounds (PIFA, PIDA), AAPH and peroxynitrite.
2. **Biological evaluation of the isolated oxidized hydroxycinnamic acid derivatives.** Bioactivity of the oxidized metabolites in comparison with their parent compounds was planned to investigate in research collaborations.
3. **Investigation of the formation of hydroxycinnamic acid metabolites in biorelevant environment.** The possible formation of the identified metabolites upon free radical scavenging by HCAs was studied by various *in vitro* and *in silico* techniques.

Materials and Methods

Starting materials and reagents: Caffeic acid, *p*-coumaric acid, methyl *p*-coumarate, AAPH and the hypervalent iodine reagents, PIFA (Bis[(trifluoroacetoxy)idodo]benzene) and PIDA ((Diacetoxyiodo)benzene) were purchased from Sigma Aldrich (Munich, Germany).

Procedures for chromatographic purification: The different oxidized metabolites were purified by combined chromatographic techniques; reversed-phase flash chromatography, and/or reversed-phase high-performance liquid chromatography (RP-HPLC), and supercritical fluid chromatography (SFC).

Procedures for structure elucidation: The structure of the obtained compounds was elucidated by means of different spectroscopic methods; 1D and 2D NMR, HR-MS, and MS-MS.

Biological evaluation of the oxidized hydroxycinnamic acid derivatives: Bioactivity testing was performed in research collaboration. The obtained methyl *p*-coumarate (**pcm**) derivatives were tested for their *in vitro* cytotoxic activity on a mouse T-cell lymphoma cell line (L5178) and its multidrug resistant counterpart ($L5178_{B1}$) obtained by transfection with pHa MDR1/A retrovirus to express the human ABCB1 transporter, using MTT assay. Two **pcm** metabolites were also tested for their cytotoxic activity on human gynaecological cancer cell lines; HeLa, SiHa, MCF-7, and lung carcinoma cell lines; a highly metastatic large cell lung carcinoma NCI-661, A549 adenocarcinoma, a non-small cell lung carcinoma NCI-H460 and its multi-drug resistant cell line pair NCI-H460/R obtained from the sensitive cell line after 3 months-long

adaptation to doxorubicin. IC_{50} values were calculated with the help of GraphPad Prism using nonlinear regression analysis.

Cell death induction by the above mentioned two metabolites was also analysed on human lung carcinoma cell lines (NCI-H460, NCI-H460/R, A549, NCI-H661) and normal human keratinocytes (HaCaT). AV/PI labelling was used to determine the percentages of apoptotic, necrotic and viable cells.

The DNA damaging effect of these two compounds was examined by Histone 2A.X relative expression, and the ROS (reactive oxygen species) production of them was detected according to dihydroethidium fluorescence, measured on CyFlow Space flow cytometer.

The effect of methyl *p*-coumarate (**pcm**) and its most effective derivative (graviquinone) on DNA damage response was evaluated by Western immunoblotting after UV irradiation (10 J/m^2) of MCF-7 cells. Images were recorded with a LAS-4000 luminescent image analyser system.

Methyl caffeate (**cm**) and its oxidized derivative were tested for their cytotoxic activity on human adherent gynaecological cancer cell lines; HeLa, SiHa, MCF-7, MDA-MB-231 by MTT assay.

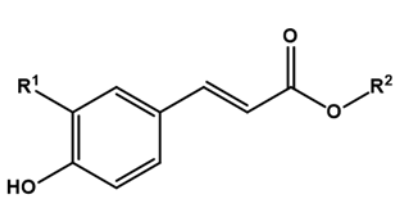
The cytotoxic activity of methyl caffeate (**cm**) was also tested with or without the presence of *tert*-butyl hydroperoxide (*t*-BHP) by MTT assay. To evaluate the effect of *t*-BHP-induced oxidative stress on the efficacy of **cm**, cells were pre-treated for 24h with medium only, or medium containing *t*-BHP at its 1/3 $IC_{50}^{(72h)}$ or $IC_{50}^{(72h)}$ concentration.

Procedure for *in silico* studies: Density functional theory (DFT) calculations were performed with the Gaussian09 package (Rev. A) in research collaboration. Mulliken spin density visualization has been carried out with the help of VMD program.

Results and discussion

Preparation of hydroxycinnamic acid esters as starting materials

To avoid decomposition and potential chromatographic challenges connected to the acid function, ester derivatives of HCAs were prepared and used in the oxidations. Methyl-, ethyl-, butyl- and isopropyl esters of *p*-coumaric acid (**1-4**) were prepared by Fisher esterification, and methyl ester of caffeic acid (**5**) was prepared with Dowex cation exchange resin. Compounds **1**-**5** were used as starting materials in our reactions, which structures are represented in Figure 1. We used for methyl *p*-coumarate (**1**) **pcm** and for methyl caffeate (**5**) **cm** abbreviations for the easier understanding.



	R^1	R^2
pcm (1):	-	Me
2:	-	Et
3:	-	But
4:	-	*i*Pr
cm (5):	OH	Me

Figure 1. Preparation of hydroxycinnamic acid esters.

Oxidation of methyl *p*-coumarate

With the aim to see whether or not more effective compounds can be formed from methyl *p*-coumarate through free radical scavenging, the effect of various oxidative conditions on the cytotoxic activity of **pcm** was investigated. A series of reaction mixtures were prepared by oxidizing **pcm** with hypervalent iodine (III) reagent, PIFA in acetonitrile, in a mixture of acetonitrile and water (9:1, v/v), in methanol, in a mixture of methanol and water (9:1, v/v), or with pyridinium chlorochromate (PCC) in dichloromethane. The obtained product extracts (OX 1-5) and the starting material **pcm** were tested for their cytotoxic activity on a mouse lymphoma cell line pair (L5178 and $L5178_{B1}$). In comparison with **pcm**, OX1 and OX2 demonstrated a highly increased cytotoxic activity, hence these reaction conditions were chosen to apply in scale-up reactions. Altogether 5 oxidized metabolites (**5-9**) were isolated from three scaled-up reaction mixtures by combined chromatographic techniques. Structures of the oxidized metabolites of **pcm** are presented in Figure 2.

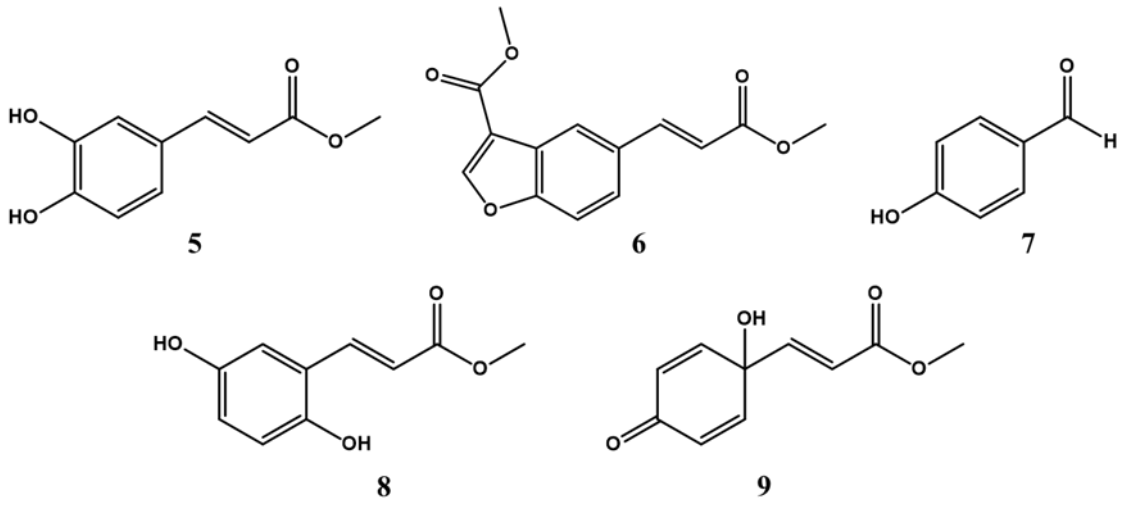


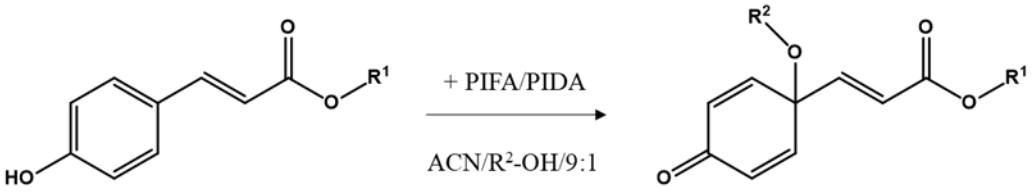
Figure 2. Chemical structures of the isolated oxidized metabolites from the reaction of **pcm** with hypervalent iodine reagents.

The formation of compound **6** likely took place similarly to the formation of dihydrobenzofurane dimers as described before, which was then followed by the elimination of

a phenol group and the formation of benzofurane skeleton. In compound **8** (methyl grevillate), that has been formerly isolated from *Grevillea robusta* A. CUNN, and from *Murraya paniculata*, the hydroxyl group unexpectedly migrated from para- to meta-position. Compound **9** (graviquinone) has also been isolated previously from *G. robusta*, and its i*n vitro* cytotoxic effect against MCF-7, NCI-H460 and SF-268 cell lines has been published.

Preparation of dearomatized *p*-coumaric acid derivatives with hypervalent iodine reagents: *p*-quinols and their *O*-alkyl ethers

A set of antitumor 1′-*O*-alkyl protoflavone analogues were previously prepared by our research group, of which a butyl ether derivative was found to exert stronger activity against cancer cell lines (Hep3B, MCF-7 and MDA-MB-231) than protoapigenone itself. Considering these favourable results in pharmacological activity and the potent antitumor effect of compound **9**, a protoflavone-inspired set of *p*-quinol and *O*-alkyl derivatives were prepared of the *p*-coumaric acid esters with hypervalent iodine reagents (PIFA, PIDA) in a mixture of acetonitrile and water (9:1, v/v) or acetonitrile and *n*-butanol or propargyl-alcohol (9:1, v/v). The structures of the 4 non-substituted *p*-quinols (**9-12**) and the 4-4 *O*-substituted butyl and propargyl ether analogues (**13-20**) are presented in Figure 3.



	R¹	R²
9:	Me	H
10:	Et	H
11:	But	H
12:	*i*Pr	H

	R¹	R²
13:	Me	But
14:	Me	Propargyl
15:	Et	But
16:	Et	Propargyl

	R¹	R²
17:	But	But
18:	But	Propargyl
19:	*i*Pr	But
20:	*i*Pr	Propargyl

Figure 3. Oxidative dearomatization of *p*-coumaric acid esters with hypervalent iodine reagents yielding *p*-quinols or their *O*-alkyl ethers.

Preparation of peroxynitrite- and AAPH-oxidized hydroxycinnamic acid derivatives

***In situ* continuous-flow oxidation of methyl cinnamates with peroxynitrite**

First, a continuous-flow reaction (CFR) system was set up, in which peroxynitrite ($ONOO^-$) can be prepared in situ from nitrite and hydrogen peroxide similarly as it was

published before. Peroxynitrite obtained this way was then reacted with HCAs. This reaction with **pcm** or **cm** in the CFR system is represented on a schematic illustration in Figure 4.

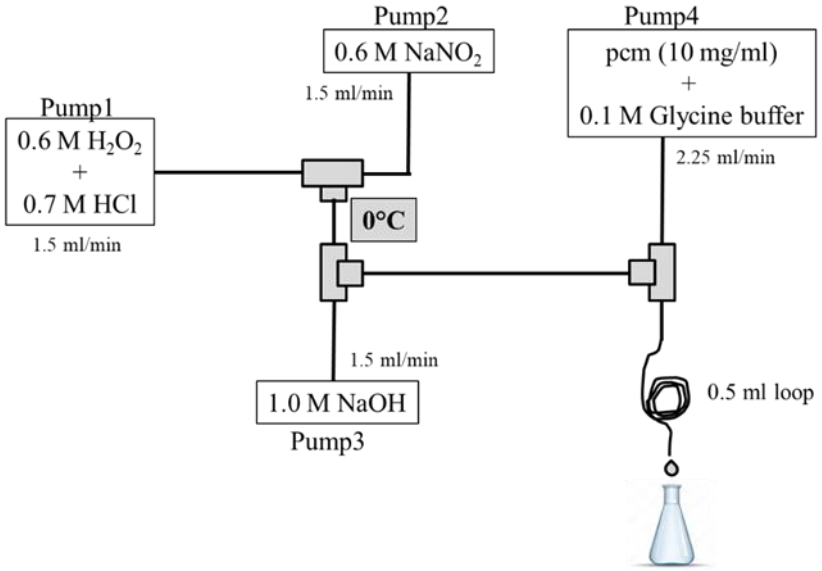


Figure 4. Experimental setup of the CFR system.

With this method, altogether 4 oxidized products (**7, 21-23**) were prepared from the reaction of **pcm** with the in situ formed peroxynitrite, and purified with the help of preparative HPLC. Structures of the oxidized metabolites (**7, 21-23**) are presented in Figure 5.

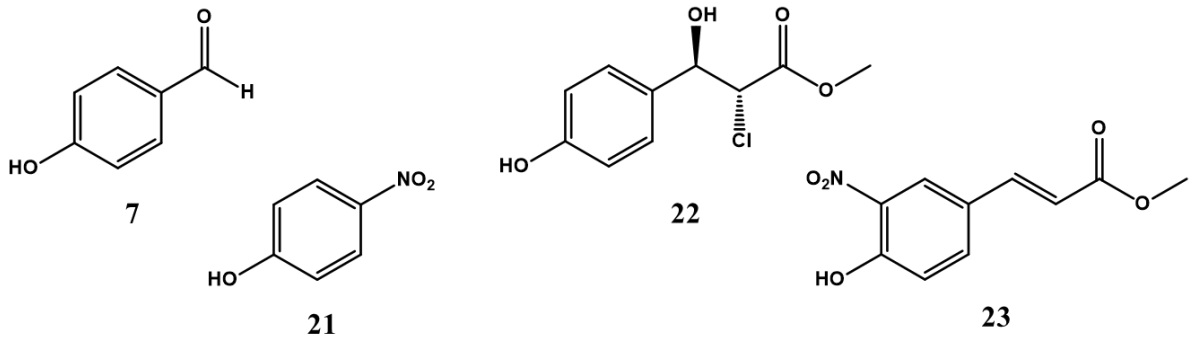


Figure 5. Structures of the peroxynitrite-oxidized metabolites (**7**, **21-23**) of **pcm**. Only one enantiomer is shown for the racemic compound **22** for clarity.

Compound **7,** i.e., *p*-hydroxybenzaldehyde, was also obtained here similarly to the reaction of **pcm** with PIFA or $^{\bullet}$OH radicals. The formation of compound **21** (*p*-nitrophenol) would be explained by a similar fragmentation and subsequent radical reaction of the aromatic ring with $ONOO^-$. Compound **22** is reported here for the first time, that is an unexpected product. It must be formed in a secondary reaction of a reactive intermediate of **pcm**, through an oxidation at the trans-olefin moiety, and a reaction with chloride ion that is a contaminant

from the preparation of $ONOO^-$. The formation of compound **23**, similarly to the case of compound **21**, represents an irreversible structural change through nitration that may also take place when a small-molecule antioxidant scavenges $ONOO^-$.

Preparation of peroxynitrite- or AAPH-oxidized crude product mixtures for bioactivity screening

We aimed to find ROS/RNS scavenging-related possible antitumor metabolites of **pcm** or **cm** in their oxidative reaction with $ONOO^-$ or AAPH. Each product mixtures were pre-purified and tested for their cytotoxic activity on human gynaecological cancer cell lines (HeLa, SiHa, MCF-7, MDA-MB-231). The reaction mixtures of **pcm** were inactive in this assay, but the reaction mixture of **cm** oxidized with AAPH showed potent cytotoxic activity, especially on HeLa cell line.

Longitudinal study and bioactivity-guided isolation of AAPH-oxidized metabolites of methyl caffeate

The oxidation of methyl caffeate (**cm**) with AAPH was selected for a longitudinal study to find the best reaction time for the isolation of the cytotoxic compound(s). Samples were taken out of the reaction mixture at specified times (i.e., 0, 1, 4, 8, 24, 30 and 48 hours) and analysed by HPLC and SFC. In parallel with the analytical measurements, the samples were tested for their cytotoxic activity on HeLa cell line. As the amount of the starting material (**cm**) decreased and the amounts of the products increased, the cytotoxic activity was gradually increasing until 24 hours, then it was decreasing. The cytotoxic activity, expressed in **cm** equivalents, showed strong correlation with the area under the curve (AUC) value of the peak of one product (**24**) present in the mixtures. The most important results of this study are summarized in Figure 6.

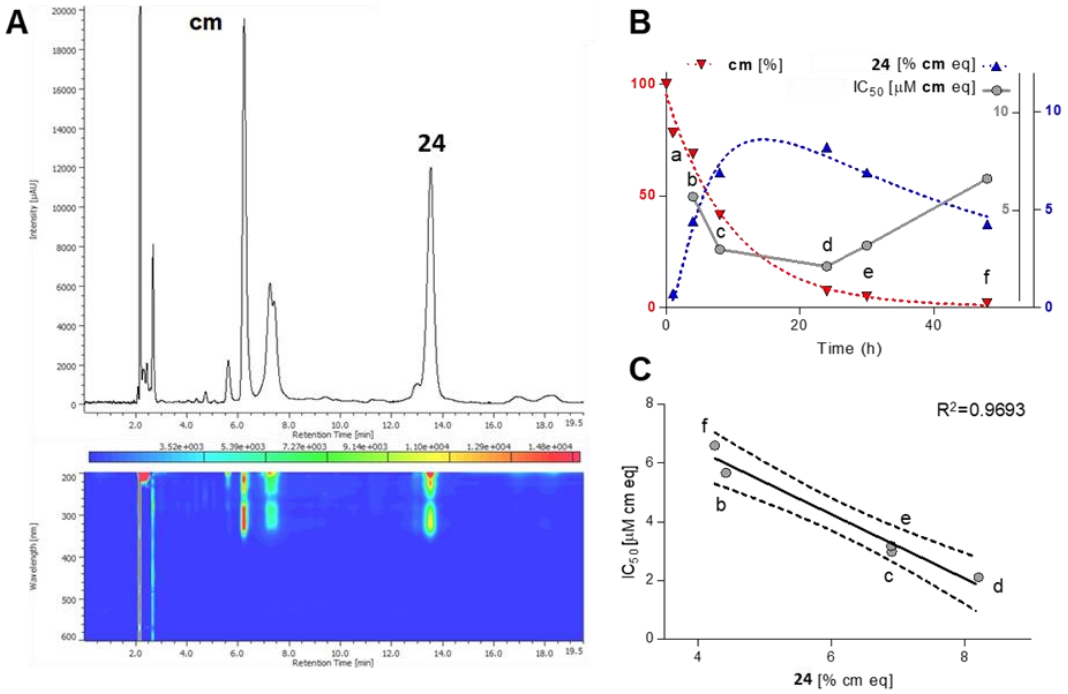


Figure 6. Longitudinal study on the reaction of **cm** with AAPH. (**A**) Supercritical fluid chromatography-photodiode array (SCF-PDA) fingerprint of the most potent cytotoxic sample taken at 24 h of reaction time. (**B**) Time dependency of the reaction and the IC_{50} values of the respective samples taken at 0 h (t_0), 1 h (a), 4 h (b), 8 h (c), 24 h (d), 30 h (e) and 48 h (f) on HeLa cells. (**C**) Linear correlation between the relative area under the curve (AUC) values of compound **24** and the IC_{50} values. The 95 % confidence interval of the regression line is shown with dashed lines, AUC is given in % relative to that of **cm** at t_0.

The 24-hour reaction time was selected for a scale-up to isolate the effective compound(s), and a bioactivity-guided purification strategy was applied. At first, the reaction mixture was separated to main fractions by preparative HPLC, and the cytotoxic activity of them was investigated on HeLa cells. From fraction 3, that showed ~80 % inhibition at 1.8 µM concentration (expressed in cm equivalents), compound **24** was purified with the help of HPLC. From fraction 5, compound **25** as a major compound was purified by SFC. Structures of compound **24** and **25** are illustrated in Figure 7.

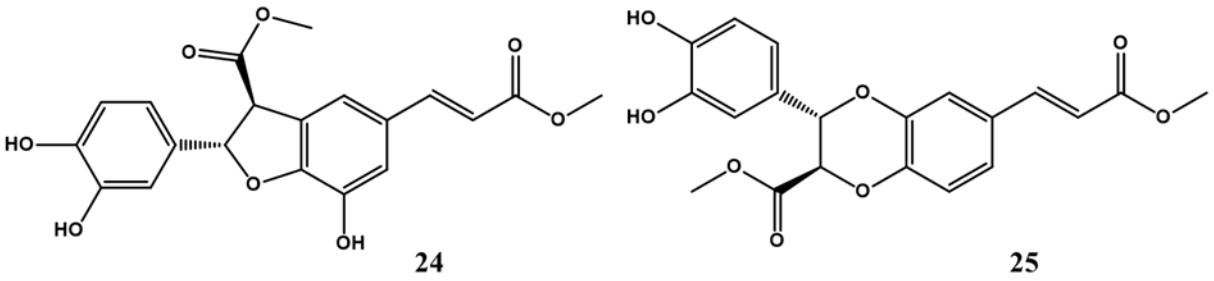


Figure 7. Structures of oxidized products (**24, 25**) isolated from the reaction between **cm** and AAPH. Both compounds were obtained as racemates; only one enantiomer is shown for clarity.

It is worth mentioning here that compound **24** was also detectable in the reaction of **cm** with peroxynitrite.

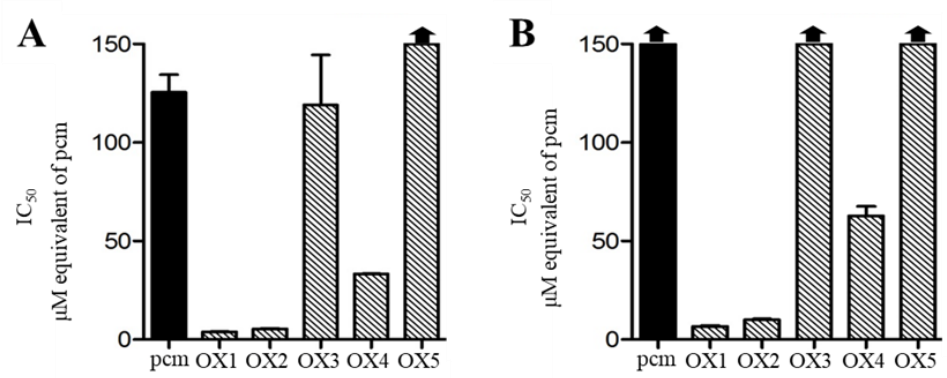
Biological evaluation of the isolated oxidized hydroxycinnamic acid derivatives

Antitumor potential of the oxidized methyl *p*-coumarate derivatives

In vitro cytotoxicity

A series of oxidized reaction mixtures (OX1-5) of methyl *p*-coumarate (**pcm**) were pre-purified and tested for cytotoxic activity on a mouse lymphoma cell line (L5178) and its multidrug resistant counterpart ($L5178_{B1}$), and two product mixtures, OX1 and OX2 showed increased cytotoxic activity in comparison with **pcm**.

Pcm and its oxidized derivatives (**5-9**) isolated from the scaled-up reactions were also tested for their *in vitro* cytotoxic activity on the mouse lymphoma cell line pair (L5178, $L5178_{B1}$). Furthermore, compounds **8** and **9** were tested on a diverse panel of human cancer cell lines; MCF-7, HeLa, SiHa, A549, lung carcinoma cell lines *e.g.*, NCI-H661, A549, and a non-small cell lung carcinoma cell line NCI-H460 and its multi drug resistant pair NCI-H460/R. Results of the cytotoxic bioassays are summarized in Figure 8.



C

	IC_{50} ± SEM (µM)									
	L5178	$L5178_{B1}$	NCI-H460	NCI-H460/R	A549	NCI-H661	HaCaT	MCF-7	HeLa	SiHa
pcm	125.60 ± 8.89	> 150	120.01 ± 2.14	1043.13 ± 59.36	322.92 ± 8.09	289.21 ± 8.42	311.22 ± 3.75	> 400	> 150	> 150
8	5.23 ± 0.29	12.38 ± 0.23	3.77 ± 0.07	7.05 ± 0.13	4.14 ± 0.09	4.19 ± 0.11	9.69 ± 0.18	-	8.51 ± 1.54	-
9	0.46 ± 0.09	0.62 ± 0.08	1.85 ± 0.03	2.06 ± 0.03	4.32 ± 0.09	1.44 ± 0.02	4.05 ± 0.10	6.22 ± 1.07	9.85 ± 1.03	8.89 ± 1.09
Dox	0.41 ± 0.07	11.83 ± 0.64	0.04 ± 0.01	1.89 ± 0.25	0.55 ± 0.16	0.35 ± 0.12	0.03 ± 0.01	1.08 ± 0.01	1.21 ± 0.05	0.76 ± 0.01

Figure 8. Cytotoxic activity of **pcm** and its oxidized metabolite mixtures (OX1-5) on a mouse lymphoma cell line pair: L5178 (**A**) and $L5178_{B1}$ (**B**). *In vitro* cytotoxic activity of **pcm** and its oxidized isolated metabolites (**8, 9**) (**C**). IC_{50} values are given in µM as mean ± SEM from three parallel experiments; Dox: doxorubicin. Compounds **5-7** are not included in this table; due to their >100 µM IC_{50} values on the mouse lymphoma cell lines.

The MDR mouse lymphoma cell line showed 2.4 times cross-resistance towards compound **8**, and less than twice cross-resistance was observed in NCI-H460/R. Compound **9** could bypass resistance of both MDR cell lines. It showed good selectivity towards cancer cells, comparing with its effect on immortalized human keratinocytes.

Cell death analysis

The percentages of apoptotic, necrotic and viable cells were determined in human lung carcinoma cell lines (NCI-H460, NCI-H460/R, A549, NCI-H661) and in normal human keratinocytes (HaCaT) after treatment with compound **8** and **9** by Annexin V/PI staining. Both of the tested HCA metabolites induced cellular necrosis. Compound **9** increased the percentage of late apoptotic NCI-H661 cells from 0.95 % (untreated control) to 9.57 % (treated), and the percentage of necrotic NCI-H661 cells from 2.58 % (untreated control) to 44.82 % (treated). After treatment with the same dose of compound **9**, the percentage of viable HaCaT cells was 91.50 %, suggesting the selectivity of compound **9** towards cancer cells.

DNA damage studies

After lung carcinoma cell lines were treated with compound **8** or **9**, the expression of Histone 2A.X (H2A.X) was analysed as a marker of DNA damage. ROS production was detected according to dihydroethidium fluorescence. The potential effect of compound **9** on DNA damage response was evaluated with Western immunoblotting after UV irradiation of MCF-7 cells. Results of the DNA damage studies are represented in Figure 9.

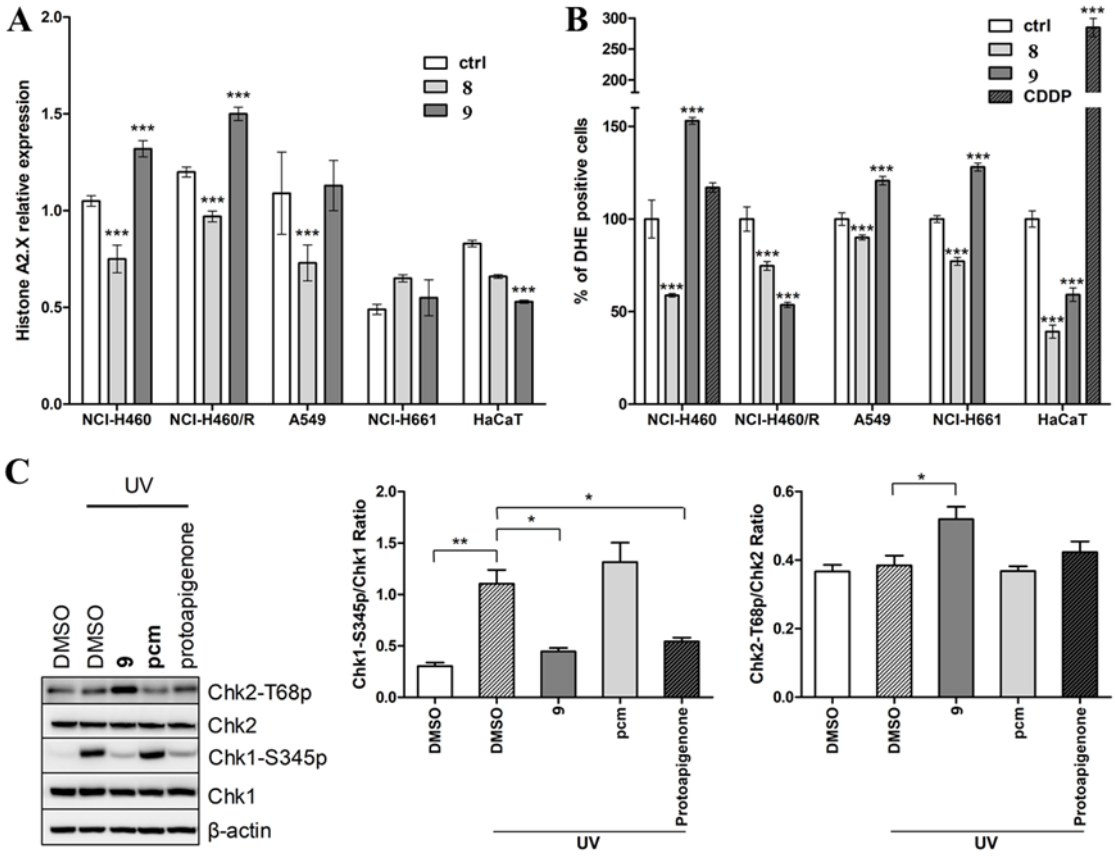


Figure 9. DNA damaging effect (**A**) and ROS production (**B**) by treatment with compound **8** or **9**. Both analyses were performed by flow cytometry, results are shown as the average ± s.d. of three independent experiments. ***: $p<0.001$. CDDP: cisplatin. The effect of compound **9** on DNA damage response (**C**). Protoapigenone was the positive control. Data represent the mean ± SD from three independent experiments; *: $p<0.05$, **: $p<0.01$.

Compound **9** induced DNA damage in the NCI-H460 cell line pair with an increased expression of H2A.X, however it was decreased in HaCaT cells, demonstrating its selectivity towards cancer cells. Compound **9** increased ROS levels in A549, NCI-H460 and NCI-H661 cells, but decreased in NCI-H460/R and HaCaT cells. A reduced expression of H2A.X and decreased level of ROS was observed in most of the cancer cell lines and also in HaCaT cells after treatment with compound **8**, suggesting its DNA protective activity.

Unlike **pcm**, compound **9** was able to modulate DNA damage response through the inhibition of Chk1-S345 phosphorylation, indicating that this compound has an ability to target cancer cells with high levels of endogenous DNA damage.

Antitumor potential of the dearomatized *p*-coumaric acid ester derivatives

The prepared *p*-quinols (**9-12**) and their *O*-alkyl ether analogues (**13-20**) were tested for their *in vitro* cytotoxic activity on a sensitive (L5178) and a multi-drug resistant ($L5178_{B1}$) mouse lymphoma cell line, and on a panel of human gynecological cancer cell lines (HeLa, SiHa, MCF-7, MDA-MB-231) as a preliminary experiment. The *p*-quinol analogue (**12**) of *p*-coumaric acid isopropyl ester showed similarly strong cytotoxic activity as compound **9** on the mouse lymphoma cell line pair. The resistance profile of the butyl ether analogues were better than that of the propargyl analogues on the mouse lymphoma cell line pair. On all the tested cancer cell lines, the *p*-quinols exerted stronger cytotoxic effect than their *O*-alkyl analogues, suggesting that such a substitution decreases the cytotoxicity of these compounds.

Cytotoxicity testing of an oxidized metabolite of methyl caffeate

In vitro cytotoxic activity of the purified compound **24** was tested on a panel of human gynecological cancer cell lines; HeLa, SiHa, MCF-7, MDA-MB-231. Results of the cytotoxicity assay are presented in Table 1.

Table 1. Cytotoxic activity of compound **24** on human gynecological cancer cell lines in comparison with its parental compound **cm**. Cisplatin was used as positive control; results were obtained from two biological replicates.

IC_{50} [95% C.I.] (µM)				
	HeLa	**SiHa**	**MCF-7**	**MDA-MB-231**
cm	450 [396.7-551.2]	> 500	175.4 [162.3-189.7]	139.3 [116.5-166.6]
24	1.1 [1.0-1.2]	> 30	1.1 [0.9-1.4]	3.9 [3.1-4.9]
cisplatin	11.7 [10.3-13.1]	13.6 [12.6-14.7]	5.2 [4.6-5.8]	25.8 [24.4-27.4]

Investigation of the formation of hydroxycinnamic acid metabolites in biorelevant environment

In vitro antitumor activity of methyl p-coumarate in the presence of H_2O_2

With the aim to investigate the effect of externally induced oxidative stress on the sensitivity of cells towards **pcm**, MCF-7 cells were treated with **pcm** in combination with H_2O_2. **Pcm** was chemically stable in presence of H_2O_2. Results of the assay are summarized in Table 2.

Table 2. Interaction between **pcm** and H_2O_2 in terms of cytotoxicity on MCF-7 cells. CI_{avg}: weighted average CI value; $CI_{avg} = (CI_{50} + 2\ CI_{75} + 3\ CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity and antagonism, respectively. Dm, m and r represent antilog of the x-intercept, slope and linear correlation coefficient of the median-effect plot, respectively.

	CI_{50}	CI_{75}	CI_{90}	Dm	m	r	CI_{avg}
2.5:1	0.79	0.60	0.45	247.66	2.109	0.985	0.556
1.25:1	0.93	0.60	0.39	250.18	3.197	0.988	0.550
0.625:1	1.06	0.68	0.44	222.79	3.610	0.974	0.623

The results showed synergism between **pcm** and H_2O_2, especially at higher rates of inhibition.

Cytotoxicity testing of methyl caffeate with or without the presence of t-BHP induced oxidative stress

Our aim was to investigate whether a possible oxidative stress-related intracellular in situ formation of compound **24** from **cm** has the chance to modulate the observed cytotoxic effect. After the cytotoxicity of *t*-BHP was determined on a panel of human gynecological cancer cell lines (HeLa, SiHa, MCF-7, MDA-MB-231) by MTT assay, a two-step combination experiment was performed. The cells were pre-treated with *t*-BHP at 1/3 $IC_{50}^{(72h)}$ or $IC_{50}^{(72h)}$ concentrations for 24 hours, then treated with **cm** (**5**) in freshly added medium and incubated for 72 hours. Cell viability was evaluated in comparison with cell controls that were treated with medium without **cm**. Results are presented in Figure 10.

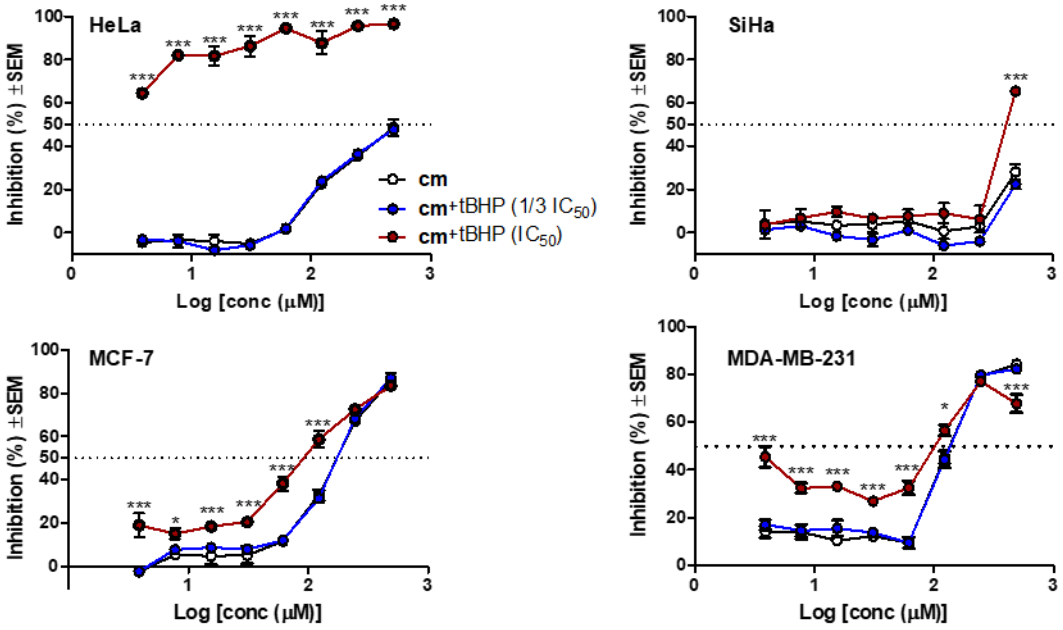


Figure 10. Cytotoxic activity of **cm** on human gynecological cancer cell lines with or without the presence of *t*-BHP induced oxidative stress. * and ***: $p<0.05$ and $p<0.001$, respectively, as compared to the single treatment with **cm**; $n=3$.

The tested cell lines were sensitized to the cytotoxicity of **cm** by the oxidative stress-inducing effect of *t*-BHP, except SiHa cells that were also the most resistant to compound **24**. The largest increase in the killing activity of **cm** was observed in HeLa cells, just like the difference between **cm** and its most effective hypothesized metabolite was larger in this cell line. Antagonism was observed in each cell line with the simultaneous co-treatment with **cm** and *t*-BHP.

Fenton reaction

Fenton reaction was performed to model the effect of oxidative stress on **pcm**, and analyse the possible metabolites upon $^{\bullet}$OH radical scavenging. Compound **9** was detectable in the Fenton reaction of **pcm** by RP-HPLC and LC-MS. Figure 11 represents the HPLC fingerprint chromatograms.

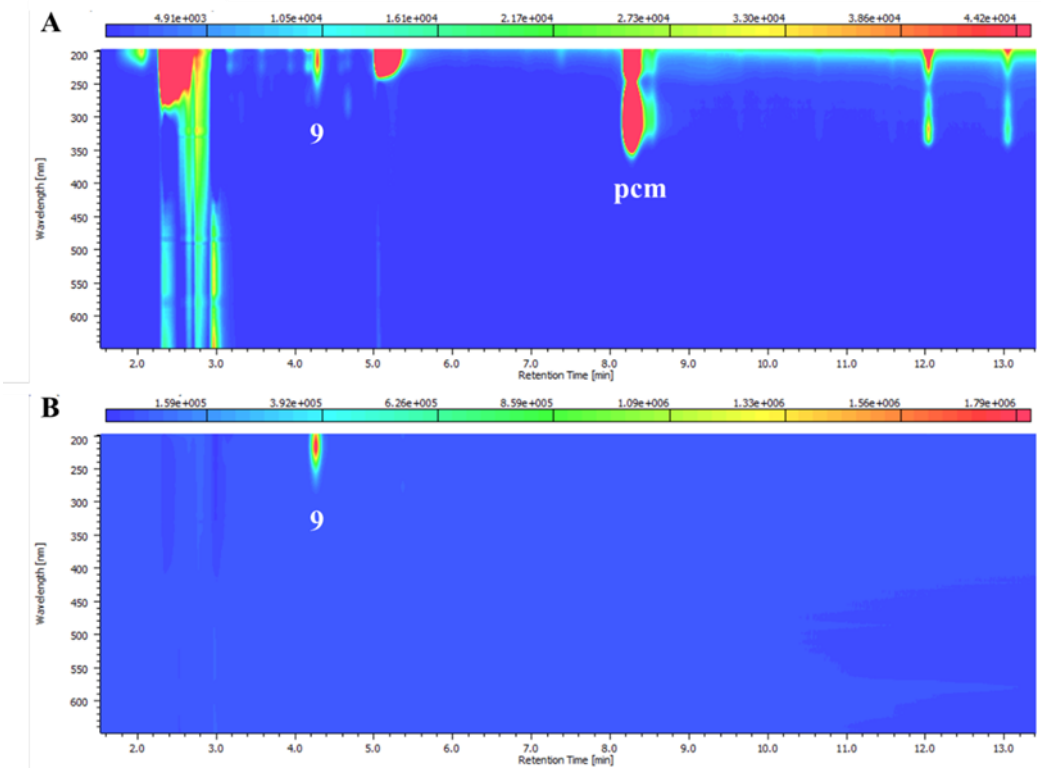


Figure 11. HPLC-PDA fingerprints of Fenton reaction of **pcm** (**A**) and compound **9** (**B**).

Hydroxyl radical scavenging activity of methyl p-coumarate

The ˙OH radical scavenging capacity of **pcm** was investigated by measuring the inhibition of the oxidative damage caused to 2-deoxyD-ribose by Fenton reaction. **Pcm** was found to be weaker scavenger than Trolox, however showed similarly strong activity as disufenton sodium, a free radical trapping antioxidant that reached phase III in clinical investigation.

In silico studies on the formation of methyl p-coumarate metabolites in the presence of ˙OH radicals

In silico studies were performed in scientific cooperation by means of density functional theory (DFT) calculations, with the aim to get deeper insight into the formation of compound **9** upon ROS scavenging. There are two possible mechanical sequential chemical pathways, in that **pcm** may be oxidized. Mechanism 1 is initiated by addition of ˙OH radicals at different positions, following by the formation of radical-adducts stabilized by H-atom abstraction. Mechanism 2 is initiated by H-atom abstraction, following by the formation of a radical that undergoes ˙OH addition at various positions, resulting in the corresponding adducts. Compound **9** can be obtained from both Mechanism 1 and 2, however compound **7** can only be obtained from Mechanism 2. Figure 12 represents the two mechanical pathways and the thermodynamic analysis.

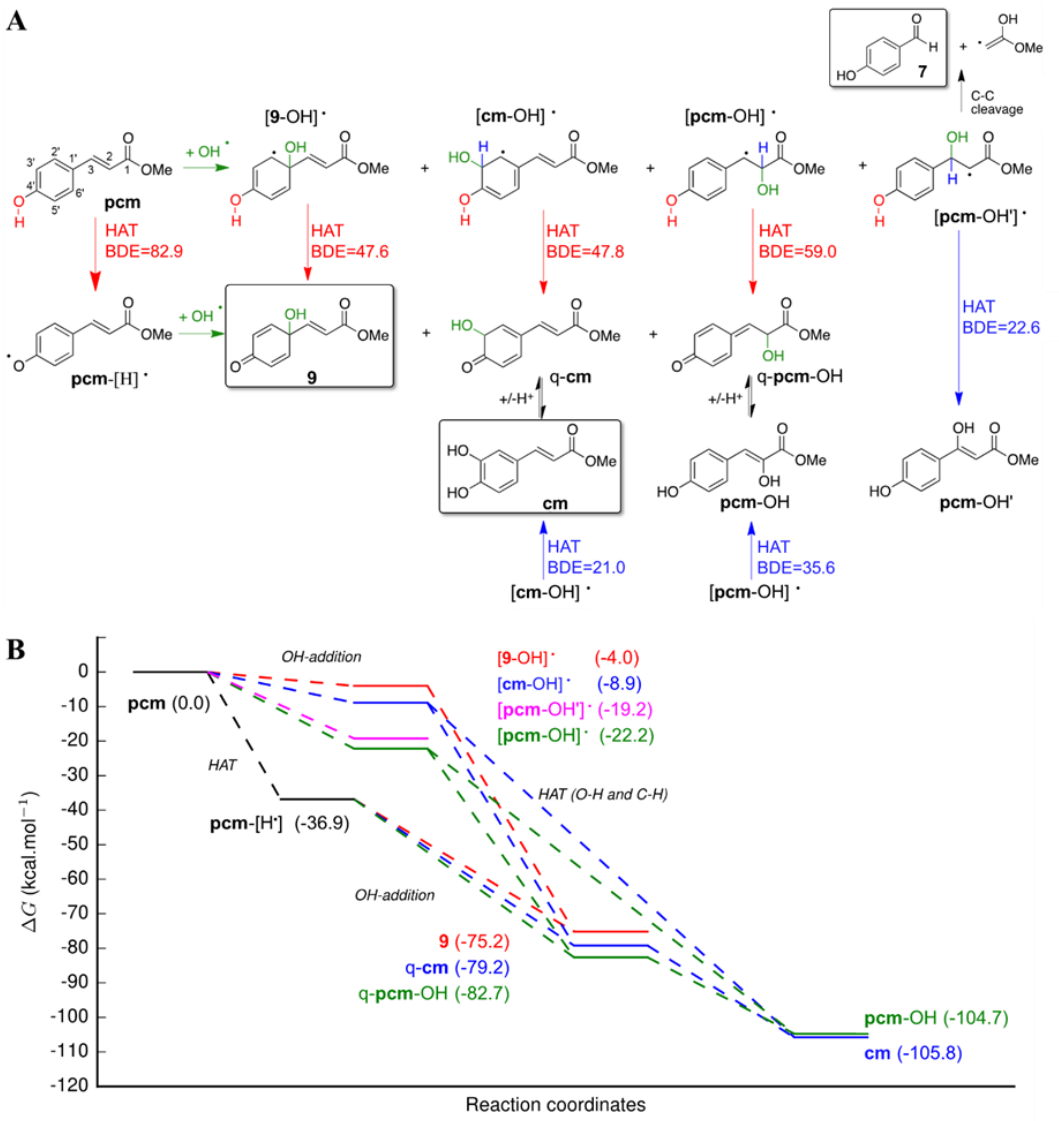


Figure 12. Oxidative transformation of **pcm** in the presence of •OH radicals (**A**) and thermodynamics of the reaction steps (**B**).

As every single step exhibits negative Gibbs energies, all the predicted compounds are thermodynamically favourable with respect to **pcm** (Figure 12B). **Cm** and **pcm**-OH are the most favourable compounds according to the calculated global thermodynamics (ΔG = -105.8 and -104.7 kcal.mol^{-1}, respectively), while compound **9** is less stable (ΔG = -75.2 kcal.mol^{-1}) as a result of dearomatization. Interestingly, only compound **7**, **cm** and compound **9** were experimentally identified among those that can be formed through these mechanisms. **Cm** is the thermodynamic product, and compound **9** is the kinetic product, suggesting the importance of kinetics in the formation of thermodynamically less favourable compounds.

Summary

- Altogether 21 oxidized hydroxycinnamic acid derivatives (**5-25**) were prepared in oxidative reactions, using different oxidizing agents, such as hypervalent iodine reagents (PIFA, PIDA), a biorelevant nitrogen species, peroxynitrite, and a peroxyl radical initiator, AAPH, and were isolated by combined chromatographic techniques.
- Two oxidized metabolites (**8**, **9**) of **pcm** showed potent antitumor effect. Compound **9** showed 2-3 orders stronger *in vitro* antitumor activity than **pcm**, and bypassed multi-drug resistance mediated by the ABCB1 transporter and by all the mechanisms present in a non-small cell lung carcinoma cell line adapted to doxorubicin. This compound demonstrated favourable tumour selectivity; it induced DNA damage in lung carcinoma cells, while exerting DNA protecting activity in normal human keratinocytes, and modulated DNA damage responses in MCF-7 cells.
- Preliminary bioactivity studies suggest that several of the 1′-*O*-alkyl ethers of related hydroxycinnamate derivatives are also promising antitumor compounds with different cell line specificity as compared to compound **9**.
- A highly potent antitumor compound (**24**), a dimerization product was identified from the oxidation of methyl caffeate (**cm**) with AAPH or peroxynitrite, i.e., it can be formed when **cm** scavenges peroxyl radicals or peroxynitrite.
- Synergism was observed in a combination treatment of MCF-7 cells with **pcm** and H_2O_2, i.e., the *in vitro* antitumor activity of **pcm** was increased in the presence of H_2O_2-induced oxidative stress.
- The H_2O_2-induced oxidative stress-related formation of compound **9** from **pcm** was confirmed by Fenton reaction. *In silico* calculations performed in research collaboration suggest compound **9** as a kinetic product of **pcm** upon $^{\bullet}$OH radical scavenging, while methyl caffeate (**cm**) as the thermodynamic product.
- The presence of *t*-BHP pre-treatment-induced oxidative stress increased the cytotoxic activity of **cm** in a way that the change of activity in different cell lines coincided with the cells' sensitivity to compound **24**. Antagonism was observed in a co-treatment of the cells with **cm** and *t*-BHP at the same time, suggesting that the increased cytotoxic activity was not due to a chemical reaction between them.
- Our results suggest that an oxidative stress-related in situ formation of new bioactive metabolites from antioxidants is possible in biological environments, and this

phenomenon can be used as the basis of discovering new bioactive compounds through diversity-oriented oxidative transformations of small molecule antioxidants.

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