α-Hexylcinnamaldehyde Synergistically Increases Doxorubicin Cytotoxicity Towards Human Cancer Cell Lines

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Abstract. *Aim: α-Hexylcinnamaldehyde (HCA), a compound derived from cinnamaldehyde, was evaluated for its potential chemosensitizing properties. Materials and Methods: The cytotoxicity of HCA was tested against Caco-2, CCRF/CEM and CEM/ADR5000 human cancer cells. Furthermore, its ability to increase doxorubicin cytotoxicity was evaluated in combination assays. Rhodamine123 efflux assay was carried out in order to highlight the possible interference of HCA with functionality of ATP-binding cassette (ABC)-transporters. Results: In spite of a low cytotoxicity, HCA increased the antiproliferative effect of doxorubicin in all the cell lines tested, being particularly effective in CCRF/CEM. The compound also reduced the rhodamine123 efflux in Caco-2 and CEM/ADR5000 cells, suggesting a possible interference with ABC transporter functionality. Conclusion: Considering that the greatest synergism between HCA and DOX was found against CCRF/CEM cells (lacking ABC pumps), it seems likely that non-specific mechanisms, including the alteration of membrane permeability, could be involved in the chemosensitizing effect of HCA.*

Chemotherapy with cytotoxic drugs represents the treatment of choice for patients diagnosed with locally advanced and metastatic cancer. Unfortunately, many oncological patients often became insensitive to a variety of structurally and mechanistically related and unrelated antitumor drugs, due to the development of multidrug resistance (MDR). MDR is defined as a multifactorial phenomenon in which both cellular (due to the alterations of the malignant cell biochemistry) and non-cellular mechanisms have been included (1). The first can be classified into non-classical and transport-based classical MDR phenotypes. Non-classical MDR mechanisms include interference with the activity of some enzymes (*e.g.* glutathione S-transferase and topoisomerase) that can reduce chemotherapy efficacy, and changes in the balance of proteins involved in apoptosis. Conversely, transport-based classical MDR mechanisms are related to the ATP-binding cassette (ABC)-transporter function (1), among which P-glycoprotein (MDR1; encoded by *ABCB1*), multidrug resistance protein (MRP1; encoded by *ABCC1*), and breast cancer resistance protein (BCRP; encoded by ABCG2) have been most extensively studied (1). These proteins are responsible for drug efflux from cells, so their overexpression in cancer cells can reduce anticancer drug bioavailability and favor MDR development. In this context, inhibiting ABC transporter function (or their expression) by weakly cytotoxic agents (namely chemosensitizers) represents a promising approach for MDR-reversion and chemotherapy success. Furthermore, in combination with a chemosensitizer, lower doses of anticancer drugs are effective, hence the adverse effects of chemotherapy can be reduced (2). In spite of their promising properties, current MDR-reversal agents (*e.g.* verapamil) are only weakly effective *in vivo* or produce too severe side-effects; therefore, new bioactive compounds are still needed (3).

Cinnamaldehyde (cinnamic aldehyde, or 3-phenyl-2-propenal), the major component (60-75%) of cinnamon bark (*Cinnamomum* spp., Lauraceae) essential oil, has been found to possess beneficial properties (including antimutagenic, antioxidative, anti-inflammatory, and antiproliferative) and has been shown to inhibit the MRP1 transporter (4, 5). In spite of these activities, the clinical application of cinnamaldehyde is limited due to its poor chemical stability and rapid clearance (half-life of about 4 min) (6). Therefore, some alkyl-derivatives have been developed in order to make the molecule less reactive and more stable. In the present study, the semisynthetic derivative α-hexylcinnamaldehyde (HCA; Figure 1), widely used as an additive in food, cosmetic and pharmaceutical industries (4), was investigated for its chemosensitizing properties in human cancer cells.

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Materials and Methods

Cell lines. Human colorectal adenocarcinoma Caco-2 and T-cell leukemia CCRF/CEM (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CEM/ADR5000 cells (a MDR1-overexpressing and doxorubicin-resistant leukemia subline kindly provided by Professor Thomas Efferth, German Cancer Research Center, Heidelberg) were used and cultivated according to El-Readi *et al.* (7).

Cytotoxicity. Cell viability was evaluated by the 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT) assay (7) and determined as follow:

$$\text{Viability } (\%) = [(OD_{\text{treated cells}} - OD_{\text{medium control}}) / (OD_{\text{untreated cells}} - OD_{\text{medium control}})] \times 100.$$

Combination assay. Cytotoxicity of doxorubicin (0.00005-1,000 μM) was tested, by MTT assay, in the presence of HCA, at inhibitory concentrations (IC) which induce 10% (IC_{10}) and 20% (IC_{20}) cytotoxicity, as found by the dose–response curve. The type of interaction (synergistic, additive or antagonistic) was evaluated by combination index (CI), isobologram analysis (IB), median-effect equation (r-value), and reversal ratio value (RR) (4, 8).

Rhodamine 123 efflux assay. Rhodamine 123 (Rho123) is a fluorescent dye effluxed by P-gp and MRP pumps, and is accumulated in cells in the presence of ABC transporter inhibitor. This assay was carried out according to El-Readi *et al.* (7) using verapamil (20 μM) as a standard P-gp and MRP1 inhibitor (9).

Statistical analysis. GraphPad Prism™ (Version 4.00) software (GraphPad Software, Inc., San Diego, CA, USA) was used. All data are expressed as the mean±standard error of the mean. Each treatment was tested in triplicate in each experiment, which in turn was carried out about three times. One-way analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post test, was used to analyze the difference between treatments. The concentration–response curves were obtained by the Hill equation, according to the software instructions.

Results

Cytotoxicity. Under our experimental conditions, HCA significantly reduced cell viability although at much higher concentrations than those of the positive control doxorubicin (Table I).

Combination assay. The combination of doxorubicin and HCA significantly reduced cell viability and increased the cytotoxicity of doxorubicin, as shown by the reversal ratio analysis (Table I). In particular, for Caco-2 cells, the IC_{50} value for doxorubicin was statistically reduced by about 3- and 7-fold in combination with HCA at IC_{10} (100 μM) and IC_{20} (181 μM) concentrations, respectively (Figure 2A, Table I). Likewise, for CCRF/CEM cells, the cytotoxic potency of doxorubicin was significantly increased in combination with HCA: the IC_{50} value of doxorubicin was reduced by about 5- and 50-fold in combination with IC_{10} (50 μM) and IC_{20} (100 μM) concentrations of HCA (Figure 2B and Table I). Viability of the resistant CEM/ADR5000 subline was also significantly affected by the combination (Figure 2C), with an increase of doxorubicin cytotoxicity of approximately 4- and 8-fold in combination with the IC_{10} (100 μM) and IC_{20} (141 μM) concentrations of HCA (Table I). According to CI, the isobologram analysis highlighted a synergistic interaction between HCA and doxorubicin for all the cell lines tested (Figure 3).

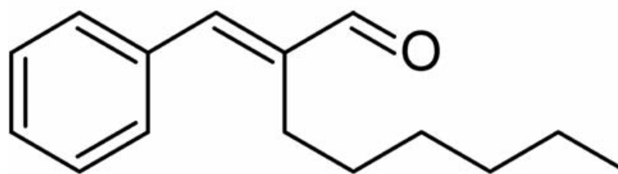

Figure 1. *Chemical structure of α-hexylcinnamaldehyde.*

Rhodamine 123 efflux assay. In Caco-2 cells, HCA increased Rho123 fluorescence in a statistically significant and concentration-dependent manner with respect to the negative control. At the same concentration of the positive control verapamil (20 μM), about 35% increase in fluorescence intensity was found. This highlights that a greater amount of Rho123 was retained in the cell, likely due to the inhibition of the efflux pumps by HCA (Figure 4A). Conversely, Rho123 efflux was not affected by HCA in the CCRF/CEM cell line (Figure 4B). A fluorescence increase, similar to that of verapamil, was observed in CEM/ADR5000 cells, thus suggesting possible P-gp pump inhibition (Figure 4C).

Discussion

Cinnamaldehyde has been found to possess beneficial properties for human health, but its clinical application is limited due to its poor chemical stability and rapid clearance (6). Therefore, some alkyl-derivatives of cinnamaldehyde have been developed in order to make the molecule less reactive and more stable. In the present study, the semisynthetic derivative HCA was investigated for its potential chemosensitizing properties in human cancer cells.

The present results show that HCA itself has a low cytotoxicity against the cancer cells tested: in contrast, cinnamaldehyde and its 2'-modified analogs were found to possess interesting antiproliferative activities (10). From a structural point of view, these molecules are characterized by the presence of a propenal in their cinnamal skeleton that forms a Michael acceptor group (11). A number of compounds containing a Michael acceptor group have been shown to

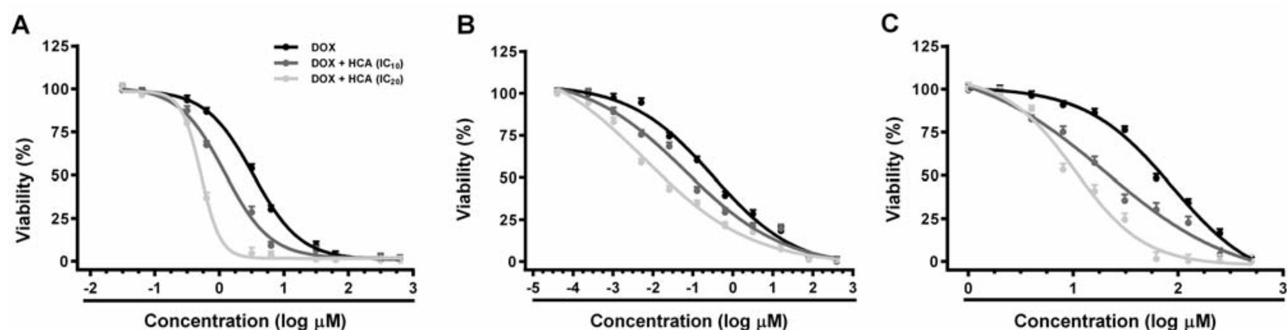

Figure 2. *Concentration–response curves for the cytotoxicity of doxorubicin (DOX) alone (black line) and in combination with α-hexylcinnamaldehyde (HCA) at inhibitory concentrations* $(IC)_{10}$ *and* IC_{20} *against Caco-2 (A), CCRF/CEM (B) and CEM/ADR5000 (C) cells. Values are expressed as the mean±SEM (n=9).*

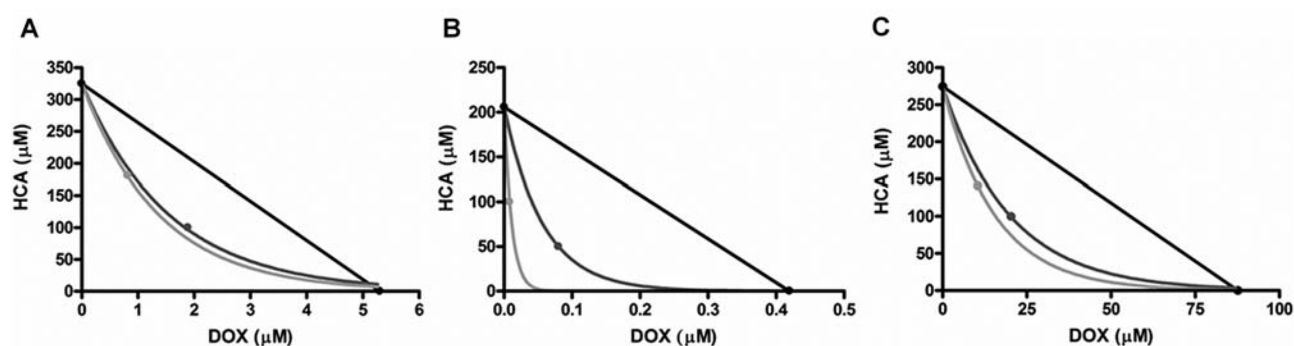

Figure 3. *Isobologram analyses of the combination of doxorubicin (DOX) with with α-hexylcinnamaldehyde (HCA) at inhibitory concentrations* $(IC)_{10}$ *(dark gray line) and* IC_{20} *(light gray line) against Caco-2 (A), CCRF/CEM (B) and CEM/ADR5000 (C) cells. The* IC_{50} *concentrations of DOX and HCA are plotted on the x and y axes, respectively. The line connecting these two points represents the additive effect; points located below and above the line indicate a synergistic and antagonistic effect, respectively.*

Table I. *Quantitation of synergistic, additive, and antagonistic interactions of the combination of α-hexylcinnamaldehyde (HCA) at inhibitory concentrations* $(IC)_{10}$ *and* IC_{20} *(μM) with the anticancer drug doxorubicin (DOX) against Caco-2, CCRF/CEM and CEM/ADR5000 cells. For each experiment, the values for replicates were averaged and the values were expressed as the mean±SEM (n=9).*

	Caco-2			CCRF/CEM			CEM/ADR5000		
Drug	IC_{50} (RR)	CI	r	IC_{50} (RR)	CI	r	IC_{50} (RR)	CI	r
HCA	325.2±1.12	-	0.99	205.7±1.18	-	0.99	274.7±1.10	-	0.99
DOX	5.3±0.50	-	0.99	0.4±0.01	-	0.99	87.7±1.2	-	0.99
DOX + HCA IC_{10}	1.9±0.16* (2.8±0.14)	0.7±0.01	0.97	0.08±0.02* (5.0±0.20)	0.44±0.004	0.95	20.3±0.13* (4.3±0.36)	0.6±0.01	0.97
DOX + HCA IC_{20}	0.8±0.06* (6.5±0.52)	0.7±0.02	0.94	0.008±0.0003* (50.9±1.58)	0.51±0.01	0.97	10.4±0.07* (8.4±0.30)	0.6±0.01	0.97

RR: Reversal ratio, the ratio between the IC_{50} for DOX alone and DOX plus HCA; CI: combination index: 1, additive effect; <1, synergism; >1, antagonism; r: correlation coefficient for the regression line, calculated from the median-effect plots according to the basic mass-action principle (4).*Significantly different from the IC_{50} for DOX alone (p<0.01; ANOVA + Dunnett's comparison multiple post test).

possess anticancer activity by interacting with cysteine residues in different cellular targets: the reactivity of the Michael acceptor moiety and lipophilicity have been proposed as structural features for their anticancer properties (12). In the semi-synthetic derivative HCA, the inclusion of an alkyl chain at α-β unsaturated double bond reduces the molecule's reactivity due to the inductive effect of the alkyl group, which stabilizes the negative charge in the intermediate carbanion. Accordingly, the presence of an α-hexyl chain in HCA stabilizes the lead compound structure (13), likely reducing the

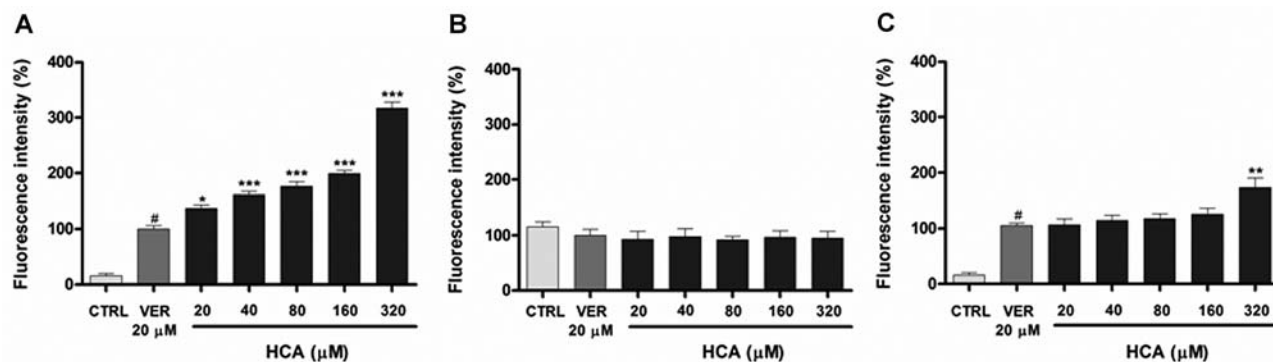

Figure 4. *Effect of α-hexylcinnamaldehyde (HCA) on rhodamine 123 efflux in Caco-2 (A), CCRF/CEM (B) and CEM/ADR5000 (C) cells. Values are expressed as the mean±SEM (n=9). VER, Verapamil. Statistically significantly different at *p<0.05, **p<0.01 and ***p<0.001 from VER, and at #p<0.001 from the negative control.*

Michael acceptor reactivity and its cytotoxicity. In contrast, these data suggest a possible role of HCA as a chemosensitizer when tested in combination with anticancer drugs, particularly doxorubicin. This compound is a P-gp substrate, hence a different cytotoxicity profile in our cell lines could be expected (14). Lacking in ABC transporters, CCRF/CEM cells are highly sensitive to doxorubicin alone because it is accumulated in these cells and its cytotoxicity is induced at very low concentrations. Conversely, in Caco-2 and CEM/ADR500 cells, the expression of ABC transporters (mainly P-gp) reduces the potency of doxorubicin, hence higher concentrations are required. Under our experimental conditions, the presence of HCA increased the potency of doxorubicin similarly in both CEM/ADR5000 and Caco-2 lines, allowing us to hypothesize that HCA acts by inhibiting the P-gp pump. However, results from Rho123 efflux assay suggest that HCA can interfere with ABC transporter functionality, although not in a pump-specific manner. Great synergism of HCA and doxorubicin was also found in CCRF/CEM cells (lacking in ABC transporters): this suggests that HCA can indirectly inhibit efflux pumps. In this context, it seems likely that non-specific mechanisms could be responsible for the reduction of ABC transporter function. In support of this, compounds containing an α,β-unsaturated moiety have been reported to modulate MRP1- and MRP2-mediated transport *via* direct (chemical interaction) or indirect (formation of glutathione conjugates which can competitively inhibit MRP1 and MRP2 or glutathione depletion) mechanisms (5). Cinnamaldehyde is also known to indirectly inhibit MRP1-mediated transport by forming glutathione conjugates (5).

Many MDR-reversing compounds have also been found to alter membrane fluidity and permeability, thereby modifying many aspects of transporters, including stability, conformation, and function: this represents a successful mechanism for reversing MDR by reducing the efflux of cytotoxic drugs. For this purpose, HCA has been found to deeply interact with a biomembrane model of dimyristoylphosphatidyl-choline multilamellar vesicles, to reduce phospholipid interaction, thereby altering the membrane properties (15). This alteration can be potentiated in the presence of other lipophilic compounds (such as doxorubicin), that can be trapped in biomembranes and interfere with both phospholipids and membrane proteins, resulting in a modulation of protein function (16). Thereafter, the inhibition of the ABC transporter function by HCA could be due to its ability to block efflux pumps as a consequence of destabilization of biomembrane structure.

In conclusion, the present results highlight that HCA possesses interesting chemosensitizing properties due to interference with ABC transporter function, possibly as a consequence of an alteration of membrane permeability, thus suggesting a possible involvement of non-classical MDR mechanisms in its chemosensitizing properties.

Acknowledgements

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