JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Cytotoxicity and Fluorescence Visualization of Ergot Alkaloids in Human Cell Lines

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ABSTRACT: The ergot alkaloids as secondary metabolites from fungi of the genus *Claviceps* are the focus of many investigations because of their pharmacological and toxicological properties. The main effects of ergot alkaloids are referred to an interaction with several receptor systems in the human body. It is well-known that ergot alkaloids are able to isomerize with one isomer being biologically active and one being only weakly active, whereas the activity is restricted to receptor interactions. Latest investigations have proven that ergot alkaloids also show cytotoxic effects and induce apoptosis in human primary cells. These effects seem to correlate with accumulation properties. It was the aim of our current study to determine such effects in cancer cell lines, because ergot derivatives are also used in tumor therapy. Our results confirm the apoptotic effects in two cancer cell lines (HepG2 and HT-29) in a high range, and accumulation measurements show an interesting correlation between the alkaloid concentration in the cell lysate of the receptor-inactive isomers and cytotoxicity. In addition, the strong accumulative effects were first visualized by fluorescence microscopy by taking advantage of the natural fluorescence properties of ergot alkaloids.

KEYWORDS: *Ergot alkaloids, cell lines, HT-29, HepG2, cytotoxicity, apoptosis, accumulation*

INTRODUCTION

Fungi of the genus *Claviceps* are well-known for their toxicological and pharmaceutical properties.[1,2] Infection of living plants leads to a formation of a resistant reservoir of the fungus, the sclerotium. This contains a variety of more than 40 secondary metabolites.[3,4] Intoxications after consumption of sclerotia-contaminated food and feed are known since first documented cases centuries ago.[1] The resulting disease, ergotism, shows different effects, ranging from vasoconstriction, gangrene of extremities, to hallucinations and spasm.[5]

The responsible substances for these effects are the ergot alkaloids. Because of structural differences, they are divided into two different groups, $\Delta^{8,9}$-ergolenes and $\Delta^{9,10}$-ergolenes.[6,7] Whereas the $\Delta^{8,9}$-ergolenes are often referred to as clavines, the group of $\Delta^{9,10}$-ergolenes are divided into lysergic acid, lysergic acid amines, and ergopeptides.[6] All of the $\Delta^{9,10}$-ergolenes have a chiral center at position C-8. Therefore, two different isomers occur in the sclerotia, 8-(*R*) and 8-(*S*) (-ine and -inine, respectively), which are widely described in the literature (Figure 1).[5−7] The distribution of both forms depends upon light, pH value, used solvent, and temperature. Both forms can easily rearrange and can be converted into each other.[8−10] This is an important aspect because the 8-(*R*) isomers are referred to as being biologically active, whereas the 8-(*S*) isomers are described to be not or only weakly active, in terms of receptor activation.[5,11] Many studies have shown a different composition of ergot alkaloids in various sclerotia samples.[12−17] Some studies reported ergotamine/-inine and ergocristine/-inine as lead alkaloids, with a nearly constant 58% of the total alkaloid content in sclerotia.[14,17] Because the ergot alkaloid composition mainly consists of $\Delta^{9,10}$-ergolenes, the European Food Safety Authority (EFSA) has focused its recent risk assessment on both forms (-ine and -inine) of ergometrine, ergotamine, ergosine, ergocristine, α- and β-ergocryptine, and ergocornine (Figure 1).[13] Toxic properties of ergot alkaloids are reported to be a result of an interaction with several neurotransmitters, because of the structural similarity, showing agonistic or antagonistic properties.[18−20] Besides this, also secondary effects were reported in the literature, e.g., induction of apoptosis in human primary cells.[21] Such secondary effects were correlated with a postulated uptake of the substances in human cells, resulting in an accumulation. It was also clearly shown that small structural differences result in different toxic properties because the different amino acid sequences in ergopeptides seem to influence their chemical and biological properties. Similar results are reported for other cells in the literature.[22,23]

The aim of this work was a continuation of the previously described monitoring of cytotoxic effects of the six main ergot alkaloids, defined by the EFSA, in human primary cells. Because our previous study has clearly shown toxic effects for ergot alkaloids in primary cells, these effects should also be studied in cell lines. With the evidence of the synthetic ergot alkaloid bromocriptine being a potent antitumor agent,[1,24,25] the impact of the six pure substances was analyzed using two different cancer cell lines. These data could be useful for further investigations concerning the toxicity of ergot alkaloids, with an identification of the most toxic compounds. Furthermore, the toxic aspects were also compared to accumulation of three model ergot alkaloids in the two different cell lines. A straightforward approach was used to visualize substance accumulation in cells without the usage of any antibodies,

Received: October 25, 2012
Revised: December 19, 2012
Accepted: December 20, 2012
Published: December 20, 2012

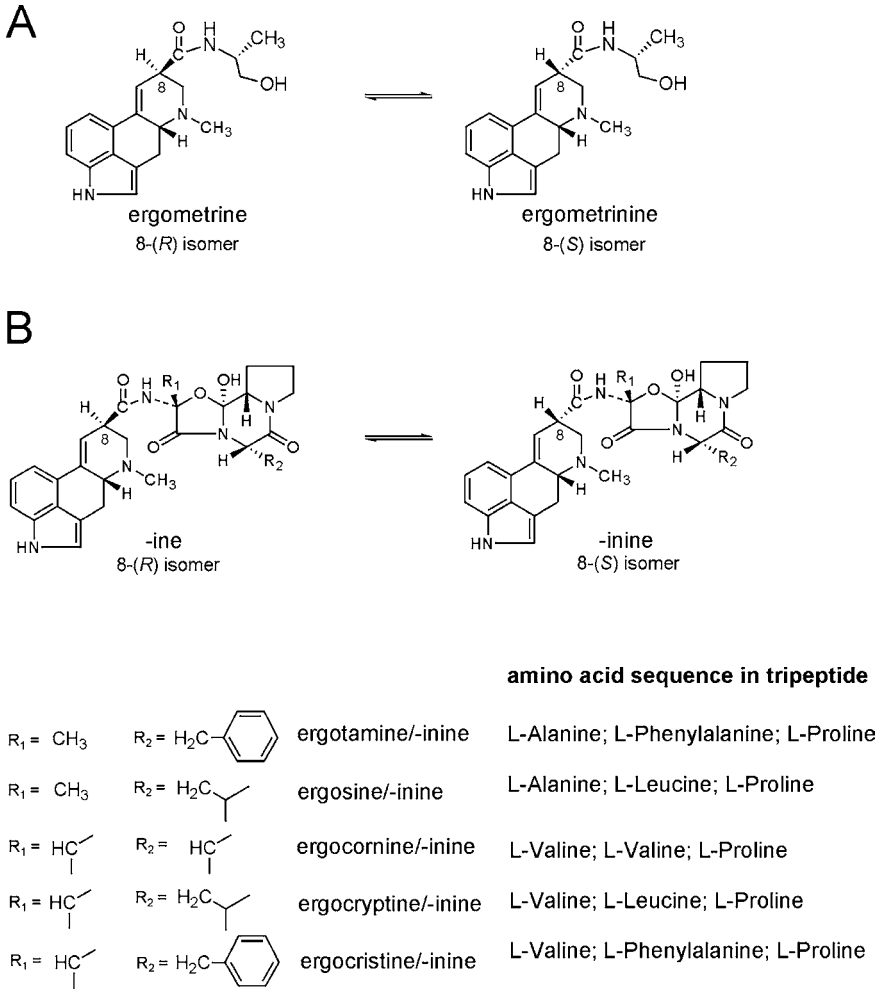

Figure 1. Structure of analyzed ergot alkaloids. (A) Lysergic acid amide ergometrine [8-(*R*) isomer] with corresponding -inine form [8-(*S*) isomer] and structures of investigated peptide ergot alkaloids (B) ergotamine, ergosine, ergocornine, α-ergocryptine, and ergocristine with corresponding -inine forms and amino acid sequence in the tripeptide structure element.

only taking advantage of the natural fluorescence properties of the alkaloids.

MATERIALS AND METHODS

Chemicals. Cell culture media, supplements, and other chemical substances were purchased from PAA Laboratories (Pasching, Austria), Biochrom AG (Berlin, Germany), Merck (Darmstadt, Germany), and Sigma-Aldrich (Steinheim, Germany).

Standard substances of ergot alkaloids were mostly obtained from Alfarma (Černošice, Czech Republic) with the exception of ergocornine, α-ergocryptine, ergotamine-D-tartrate, and ergometrine-maleate, which were from Sigma-Aldrich. Ergosine and T-2 toxin were both isolated and purified in our lab.[26] Investigated ergot alkaloids were analyzed to determine the distribution of (*R*) and (*S*) isomers. All of the standard alkaloids were nearly only consistent of (*R*) isomeric ergot alkaloids (≥95%). The internal standard methysergid-maleate was purchased from Biotrend (Wangen, Switzerland). Substances were stored as 2 mM stock solutions in ethanol/0.25 g/L tartaric acid (40:60, v/v) to guarantee stability.[8]

Cell Culture. The cultivation medium of the investigated cell lines, human colon carcinoma cell line (HT-29) and the human hepatocellular liver carcinoma cell line (HepG2), was Dulbecco's modified Eagle's medium (DMEM) with the addition of 1% non-essential amino acids, 1% penicillin/streptomycin/glutamine, and 10% fetal calf serum (FCS). Both cell types were subcultured twice a week, after reaching 90% confluency with a ratio of 1:10, or were used for experiments, described in individual sections.

Incubation experiments with investigated ergot alkaloids or T-2 toxin were performed using DMEM/Ham's F-12 medium, with the addition of 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Concentrations of ergot alkaloids containing solutions were corrected by previously described correction factors, to ensure the use of the proper concentration of the 8-(*R*) isomer, because of the rapid formation of an equilibrium between the two isomers -ine/-inine. For the six investigated ergot alkaloids, the used factors were ergometrine/-inine (1.4), ergocornine/-inine (2.0), ergotamine/-inine (1.8), α-ergocryptine/-inine (2.4), ergocristine/-inine (5.0), and ergosine/-inine (1.6).[21] All working steps were performed under red light conditions to minimize isomerization effects. Because of a rapid isomerization process, all substances were always incubated as a mixture from -ine and -inine forms.

For cultivation of the cells or incubation with substances, the cells were maintained at 37 °C and a 8.5% CO_2 atmosphere.

Cell Viability Determined by CCK-8. Cytotoxicity of ergot alkaloids was screened using the CCK-8 assay as described in the literature, being similar to the well-established MTT assay.[21] Briefly, cells were seeded in 96-well plates with at least 1×10^4 cells in every well and 100 μL of medium. Substances were added in a concentration range from 0.001 to 20 μM for 48 h of incubation time after the synchronization phase (maintaining 24 h in serum-free medium after seeding). After WST-8 solution was added, the absorption at 450 nm was measured with a microplate reader and the absorbance of the ergot-alkaloid-incubated wells was compared to solvent-treated control wells.

dx.doi.org/10.1021/jf304569q | J. Agric. Food Chem. 2013, 61, 462−471

Lactate Dehydrogenase (LDH) Release. As a marker for necrotic effects of substances, LDH release to cultivation medium was measured. The assay was performed according to a literature protocol as the conversion of NADH to NAD^+ was determined.[27] Cells were seeded with 1×10^5 cells in 24-well plates and treated the same way as in the CCK-8 assay. Incubated concentrations range from 0.01 to 20 μM for 24 and 48 h. Measurements were performed in a 96-well plate using 40 μL of incubation medium and 10 μL of cell lysate. The method for lysis of the cells was performed according to the literature with a volume of 100 μL of cell lysate.[28] The total volume of 200 μL was reached by adding reaction buffer in a required amount (100 mM HEPES, 0.14 g/L NADH, and 1.1 g/L sodium pyruvate at pH 7). Absorbance was measured at 355 nm at 37 °C every 2 min, and activity of LDH was calculated after determination of the ratio of LDH activity in cell medium and cell lysate (as mU/min). The total release of LDH from intra- to extracellular medium was calculated. The ergot-alkaloid-incubated samples were compared to solvent-treated control cells (expressed as a percentage of the control).

Caspase-3 Activation. For a calculation of caspase-3 activation, cell lysates of LDH release experiments were used (see the Lactate Dehydrogenase (LDH) Release section). Cell lysate was mixed in an equal amount with reaction buffer [50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT), and 80 μM *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-AFC)] in a black 96-well plate. After an incubation period of 1 h, the fluorescence activity of cleaved 7-amino-4-trifluoromethylcumarin (AFC) was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. For quantification, an AFC standard calibration curve, ranging from 0.3 to 12.8 μM, was used. The data were all correlated with the protein content of individual samples. These were determined using the same cell lysate, as described above. An amount of 5 μL was mixed with 200 μL of reaction mixture in a 96-well plate according to the bicinchoninic acid (BCA) assay kit manual from Sigma-Aldrich (Steinheim, Germany), containing BCA/4% copper sulfate (50:1, v/v). The plate was incubated for 30 min at 37 °C and maintained for 1 h in the dark at room temperature before measuring the absorbance of the formed complex at 560 nm. Quantification was performed using a calibration curve of bovine serum albumin (BSA) with a concentration ranging from 50 to 500 μg/mL.

SubG1 Formation Determined by Flow Cytometry. For cell cycle analysis, cells were seeded with 5×10^5 cells on a 12-well plate and were treated as mentioned before (see the CCK-8 Assay section). Ergot alkaloids were incubated with a concentration of 10 μM for 48 h, as well as the positive control T-2 toxin. Sample preparation was performed as described in earlier work.[21] Briefly, cell medium was collected for individual samples, and remaining cells were washed with phosphate-buffered saline (PBS) before detaching using Accutase (PAA Laboratories, Pasching, Austria). After washing, the cells were fixed using 0.5 mL of 70% ethanol (v/v) at −20 °C for 1 h. After fixation, cells were centrifuged (as before at 4 °C) and were washed with PBS. RNase was added in a final concentration of 10 μg/mL for 30 min at 37 °C. After incubation, propidium iodide was added with 25 μg/mL for DNA staining. Samples were incubated on ice and were measured within 1 h after staining with a FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany). The fluorescence parameter was 620 nm using FL-3 amplification, with at least 20 000 cells counted in relevant gates for every sample.

Accumulation of Ergot Alkaloids. Determination of an accumulation of investigated ergot alkaloids was performed using at least 1×10^5 cells in a 24-well plate. Cells were seeded, and after 24 h, 1 μM of the ergot alkaloids were incubated. Samples were collected by removing the cell medium, washing the remaining cells twice with PBS, and performing a cell lysis with 200 μL of 1% Triton X-100 solution. Chosen time points were 5, 10, 20, 30, 40, and 50 min and 1, 2, 4, 6, 8, 24, 48, and 72 h. After a centrifugal step (10000*g* for 10 min at 4 °C), 100−150 μL of supernatant was mixed with internal standard methysergid-maleate (final concentration of 162 ng/mL), and samples were analyzed with high-performance liquid chromatography with fluorescence detection (HPLC−FLD).

Separation of the samples was performed with a 250 × 4.6 mm inner diameter, 4,6 μm, Varian OmniSpher C18 column (Darmstadt, Germany). The further HPLC system consists of a binary pump (Merck-Hitachi L-7100, Tokio, Japan), an autosampler (Merck-Hitachi AS-2000A), and a fluorescence detector, adjusted to the characteristic wavelength for ergot alkaloids: excitation at 330 nm and emission at 415 nm (Merck-Hitachi FLD F-1050). The mobile phase was a combination of acetonitrile (A) and ammonium carbamate buffer 0.2 g/L (B). The volume of injected sample was 20 μL, and the flow rate of mobile phase was 1 mL/min.

Peptide ergot alkaloids ergotamine/-inine and ergocristine/-inine were separated using a gradient, with starting conditions of 60% A for 5.5 min, increasing to 80% in 2.5 min, and holding this for 2 min. Re-equilibration of the system was performed for another 4 min at starting conditions of 60% A, with a total run time of 14 min. Ergometrine/-inine was separated with a step gradient, consisting of 27% A, which was raised after 2.5 min to 32% for 2.5 min, and then equilibrating the system again for 8 min. The total run time was 13 min. Quantification of ergot alkaloids was performed by comparing the peak area to the internal standard using the data analysis software Merck-Hitachi D-7000 HSM HPLC system manager and a calibration curve ranging from 40 to 400 ng/mL. The results of the accumulation studies were all corrected with regard to the cell number and cell volume in each individual well, measured with CASY model TT for every chosen time point.

Visualization of Ergot Alkaloids by Fluorescence Microscopy. For fluorescence microscopy, 1×10^6 cells were seeded in 6-well plates. After 24 h, 5 μM ergotamine/-inine and 1 μM ergocristine/-inine solution was added for 5 h. Cell medium was removed, and remaining cells were washed once with PBS and trypsinized. The cell solution was cytocentrifuged (314*g* for 8 min at 20 °C) on object slides. Fixation of cells was performed with 4% paraformaldehyde (w/v) solution for 20 min at 4 °C. After a short drying period, 25 μL of VECTASHIELD HardSet mounting medium with DAPI (Vector Laboratories, Peterborough, U.K.) was added. The stained samples were directly used for fluorescence microscopy (Axio Imiger, M2, Zeiss, Oberkochen, Germany), with a mercury-vapor lamp and filter systems, with excitation at 365/50 nm, dichroism at 395 nm, and emission at 445/50 nm for DAPI and excitation at 470/50 nm, dichroism at 495 nm, and emission at 525/50 nm for ergot alkaloids. Pictures were evaluated with Axiovision software 4.5 (Zeiss, Göttingen, Germany).

Statistics. All presented data are given as value ± standard error of the mean (SEM). Experiments concerning cytotoxicity and accumulation were performed in three individual passages. A minimum of 6 wells were determined for CCK-8 assay per group, and a minimum of 4 wells were determined for further cytotoxicity studies, resulting in 12−18 samples. For accumulation and subG1 formation results, 2 wells were quantified for individual groups, resulting in 6 samples. Significant differences ($p \leq 0.05$) were determined using the unpaired Student's *t* test with Excel 2003. Calculation of EC_{50} values was performed using SigmaPlot 11.0 according to published data.[29]

RESULTS

Cytotoxicity. *CCK-8 Assay.* Figure 2 presents the cytotoxic effects of the six tested substances in a concentration ranging from 0.001 to 20 μM after an incubation time of 48 h. Cell viability decreases similarly for HepG2 as well as for HT-29 cells, with ergocristine showing the strongest impact, with an EC_{50} value of 1.3 μM for HepG2 and 1.7 μM for HT-29 cells (Table 1). Viability was significantly different in comparison to control cells starting at 0.5 μM, with a remaining value of 20% for the highest used ergocristine concentration. Similar curves were obtained for the other substances, with a shift to higher concentrations (EC_{50} values for HepG2; Figure 2A and Table 1): α-ergocryptine, 6.5 μM; ergocornine, 10.2 μM; and

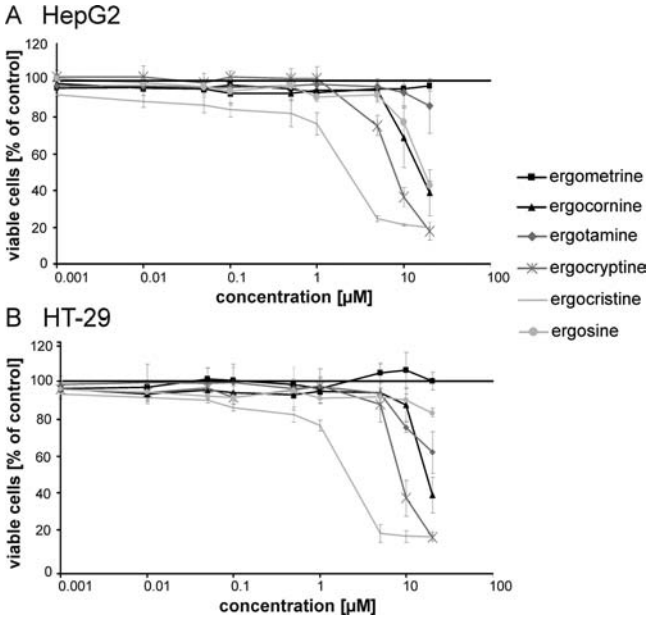

Figure 2. Cell viability of (A) HepG2 and (B) HT-29 after concentration-dependent incubation with ergot alkaloids for a period of 48 h. Number of analyzed samples (n) = 18, with significant differences ($p \leq 0.05$) for ergocristine (A, 1−20 μM; B, 0.5−20 μM), α-ergocryptine (A/B, 5−20 μM), ergocornine (A/B, 10−20 μM), ergosine (A, 10−20 μM; B, 20 μM), and ergotamine (B, 10−20 μM).

Table 1. Calculated EC_{50} Values for Tested Ergot Alkaloids[a]

	EC_{50} value (μM)	
	HepG2	HT-29
α-ergocryptine	6.5 ± 0.3	7.9 ± 0.6
ergocornine	10.2 ± 0.8	11.5 ± 1.6
ergocristine	1.3 ± 0.3	1.7 ± 0.5
ergosine	9.7 ± 1.1	nd[b]
ergometrine	nd	nd
ergotamine	nd	nd

[a]Cells were incubated for 48 h with concentrations ranging from 0.001 to 20 μM. All ergot alkaloids were applied as mixtures of the -ine/-inine form, with a corrected concentration to the -ine forms. Results are given as the mean ± SEM (n = 18). [b]nd = not detectable.

ergosine, 9.7 μM. No toxic effects were detectable using ergometrine or ergotamine. For HT-29 cells, the obtained curves and, therefore, EC_{50} values slightly differ in contrast to HepG2 cells (Figure 2B and Table 1). Ergocornine (EC_{50} = 11.5 μM) and α-ergocryptine (EC_{50} = 7.9 μM) showed similar values, and ergometrine remained with no cytotoxic effect. In contrast to the effects in HepG2 cells, ergotamine incubation resulted in a low toxic effect in HT-29 cells and ergosine showed no effect. All calculated EC_{50} values are summarized in Table 1.

Necrotic Effect Determined by LDH Release. The necrotic activity was measured using the LDH release. Figure 3 shows the toxic relevant concentrations ranging from 1 to 20 μM for an incubation time of 24 h (data for control and 0.01 and 0.1 μM not shown because no effect was observed). For HepG2 cells (Figure 3A), the previous toxic-characterized compounds ergocornine, α-ergocryptine, and ergocristine (see the CCK-8 Assay section) showed a significant effect on LDH release using 10−20 μM with 5−15% in comparison to control cells. In contrast, ergosine-incubated samples only showed a significant weak effect using 20 μM (5%). Ergotamine and ergometrine incubation had no impact on the cell viability. Using HT-29 cells (Figure 3B), ergocristine incubation showed a much stronger effect than the other investigated compounds, with about 20% LDH release in comparison to control cells using 10−20 μM after 24 h of incubation. Incubation with 10 μM ergocornine gave only a weak signal (3.5%), while significant results in the range of 3−10% could be obtained using 20 μM. Ergometrine and ergosine showed no effect in HT-29 cells. After a prolonged incubation period of 48 h (data not shown) the effects slightly increased to 10−25% for ergocristine and α-ergocryptine in HepG2 cells. For HT-29 cells, the LDH release increased dramatically after 48 h with a nearly complete disruption of all cells using ergocristine and α-ergocryptine, while the effects for ergocornine and ergotamine were only slightly enhanced.

Apoptosis Determined by Caspase-3 Activation. To elucidate further toxic effects, caspase-3 activation as a marker of apoptosis was measured after an incubation time of 24 and 48 h. To compare the results, T-2 toxin was used as a positive control, inducing apoptosis.[30,31] Figure 4 represents the results after 24 h of incubation with the six investigated ergot alkaloids in concentrations ranging from 1 to 20 μM (data for 48 h not shown). Similar to the obtained LDH release (see the Necrotic Effect Determined by LDH Release section), the same alkaloids induced an apoptotic effect. In HepG2 cells (Figure 4A), ergocristine and α-ergocryptine showed a caspase-3 activation of about 1000% in comparison to control cells using 10 or 20 μM, analogous to the positive control (10 μM T-2 toxin). The effect of ergocornine was also in a similar range using 20 μM but halved (500%) using 10 μM. Ergotamine- and ergosine-incubated samples showed an effect using 20 μM with 300−400% caspase-3 activation. In contrast to the other ergot

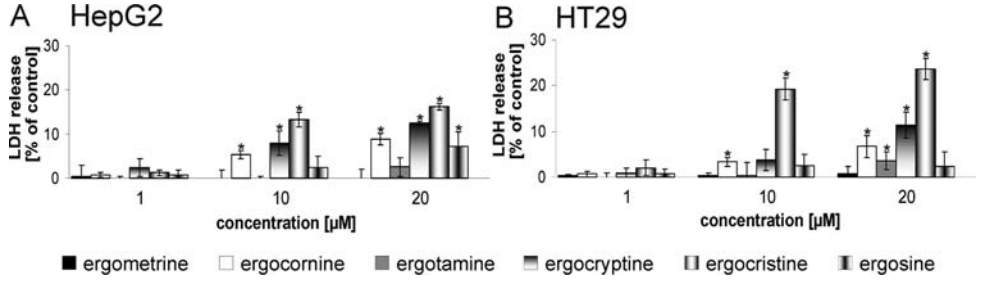

Figure 3. LDH release (in percentage of control cells, with control cells as a 0% value) after 24 h of incubation with different ergot alkaloid concentrations for (A) HepG2 and (B) HT-29. Number of analyzed samples (n) = 12, with asterisks indicating significant differences in comparison to control cells ($p \leq 0.05$).

dx.doi.org/10.1021/jf304569q | J. Agric. Food Chem. 2013, 61, 462−471

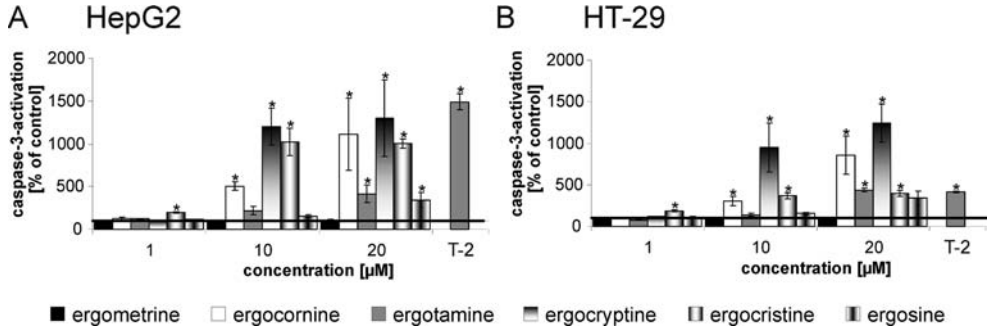

Figure 4. Concentration-dependent caspase-3 activation for (A) HepG2 and (B) HT-29 cells after 24 h of incubation with different ergot alkaloids, presented as a percentage of control cells (representing 100% value). Number of analyzed samples (n) = 12, with asterisks indicating significant differences in comparison to control cells ($p \leq 0.05$).

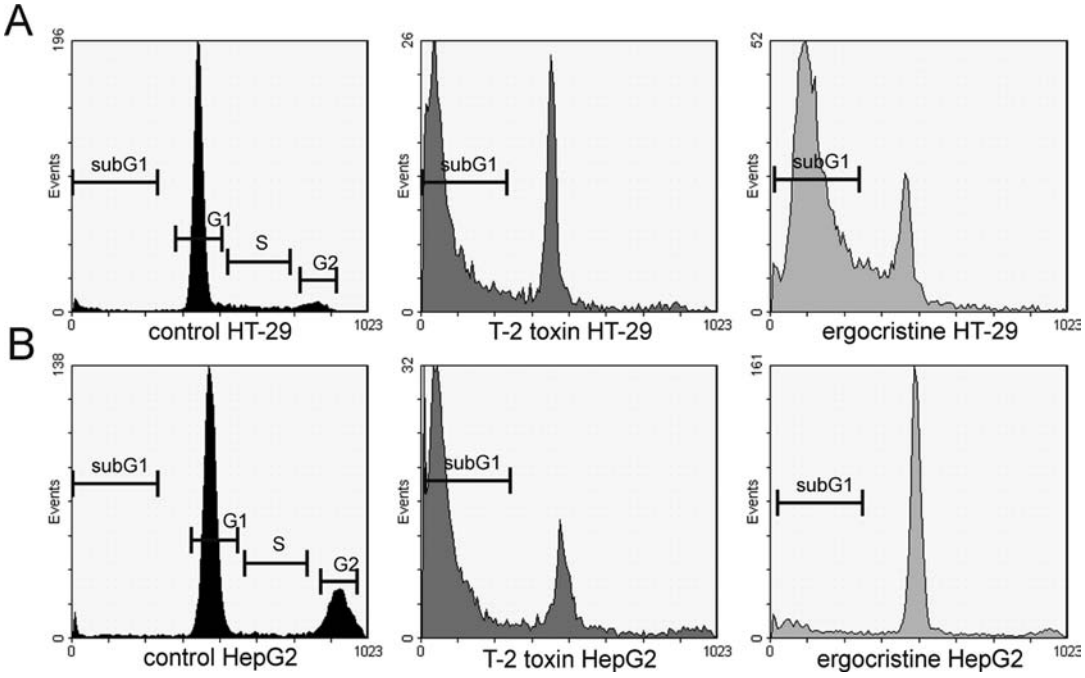

Figure 5. Histogram plots of cell cycle analysis of (A) HT-29 and (B) HepG2 cells after 48 h of incubation with 10 μM T-2 toxin and 10 μM ergocristine/-inine. Marking of complete cell cycle phase distribution in control cells and substance-incubated samples, with only the subG1 region labeled.

alkaloids, a concentration of 1 μM ergocristine resulted in a 200% caspase-3 activation.

For HT-29 cells (Figure 4B), similar results were obtained for ergocristine starting at 1 μM with about 200%, increasing to about 350% at 10 or 20 μM. The strongest activation was measured using α-ergocryptine with 1000−1200% after an incubation with 10 or 20 μM, in contrast to the positive control T-2 toxin (10 μM) with 400% caspase-3 activation. Ergocornine and ergocristine appear to have similar effects using 10 μM, but as for HepG2, a more pronounced signal was only detected using 20 μM ergocornine. The effect induced by ergocristine remained nearly constant with increasing concentrations. Ergometrine and ergosine had no impact on HT-29 cells. For an incubation time of 48 h, the tendency of lowered caspase-3 activation could be found using the highly effective substances ergocristine, ergocornine, and α-ergocryptine for both cell lines. The other compounds induced only a small increase in caspase-3 activity (data not shown).

Apoptosis Verification Using SubG1 Formation. As an additional marker for apoptosis, the formation of a subG1 peak in cell cycle analysis was measured after 48 h of incubation with 10 μM of each tested ergot alkaloid. Figure 5 presents the formation of a broad subG1 peak after the addition of T-2 toxin in both cell lines (Figure 5A, HT-29; Figure 5B, HepG2), in contrast to control cells with no peak in the relevant region. This subG1 peak formation could also be confirmed after ergot alkaloid incubation. Figure 5 shows an example of ergocristine-incubated samples (10 μM) with an intensive subG1 peak for HT-29 cells (Figure 5A) and a weak signal for HepG2 cells (Figure 5B). For quantification, the formed peak areas were compared to solvent-treated control cells. Resulting values are presented in Table 2. For HT-29 cells, ergocornine- and α-ergocryptine-treated samples showed about 800% subG1 peak formation, comparable to the positive control T-2 toxin. Ergocristine-incubated samples exceeded this with 1000% subG1 peak formation in comparison to control cells. In contrast to this, ergotamine showed a subG1 formation of about 300%, which was significantly different to control cells. The other tested substances did not show any significant differences. Effects in HepG2 cells were notably lower, with the

dx.doi.org/10.1021/jf304569q | J. Agric. Food Chem. 2013, 61, 462−471

Table 2. Formation of the SubG1 Peak after Incubation with Ergot Alkaloids (10 μM) for 48 h[a]

	HepG2 (%)	HT-29 (%)
control	100	100
T-2 toxin	847 ± 123	747 ± 68
α-ergocryptine	294 ± 45	826 ± 232
ergocornine	138 ± 12	769 ± 215
ergocristine	231 ± 63	1042 ± 80
ergosine	103 ± 6	227 ± 138
ergometrine	98 ± 5	99 ± 2
ergotamine	100 ± 11	329 ± 113

[a]Results are displayed as a percentage of the control, with the control set to 100%. Values are presented as the mean ± SEM (n = 6).

same alkaloids leading to an effect, ranging at about 130−300%. Ergosine, ergotamine, and ergometrine incubation resulted in no effect.

Cellular Accumulation. *Quantification of Ergot Alkaloids in Cell Lysate.* Accumulation of ergot alkaloids in cell lysate was determined for both individual isomers 8-(R) and 8-(S), -ine and -inine forms, respectively, because they can be easily separated with reversed C18 HPLC (see the Materials and Methods). Figure 6 displays the accumulation curves for ergotamine/-inine and ergocristine/-inine (each 1 μM) for a time period of 72 h in both investigated cell lines. Independent from the used cell line, the investigated ergot alkaloids revealed similar accumulation curves. For HepG2 cells, ergotamine (Figure 6A) and the corresponding isomer ergotaminine accumulated in the cell lysate to a maximum of 200-fold (200 μM after 6 h) and were then only slowly excluded from the cells. The same results were obtained incubating an ergocristine/-inine mixture, with ergocristine also being enriched in a similar range. In contrast to this, the ergocristinine concentration had shown an additionally rapid increase in a maximum accumulation of 600-fold of the initial concentration after 4 h. A plateau was formed with a decrease only measurable after 48 h (Figure 6B).

For HT-29 cells, the same tendency was found. Both 8-(R) isomers were detectable in nearly the same range as in HepG2 cells. Besides the enrichment of ergotamine, the enhancement of corresponding ergotaminine was slightly higher, with a maximum of 400-fold after 8 h (Figure 6C). More pronounced differences occurred for the accumulation of ergocristinine, because the same tendency was found as described before with a maximum concentration of 900-fold of the initial concentration after 8 h. In contrast to HepG2 cells, the alkaloid concentration decreased more rapidly in HT-29 cells but the alkaloids were still detectable with 200-fold accumulation after 72 h (Figure 6D).

In all experiments, applied ergometrine/ergometrinine was not detectable in cell lysate.

Fluorescence Microscopy. Visualization of accumulation was elucidated with fluorescence microscopy. Figure 7 shows the incubation of an ergotamine/-inine (5 μM) and ergocristine/-inine (1 μM) mixture for a time period of 5 h. Cell nuclei were

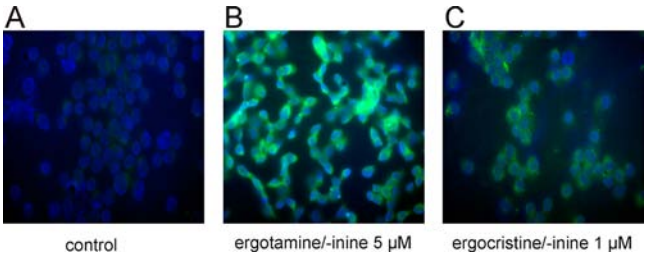

Figure 7. Fluorescence microscopy of HT-29, with (A) control cells, (B) ergotamine/-inine (5 μM), and (C) ergocristine/-inine (1 μM) incubated samples for 5 h. Cell nuclei were stained with VECTASHIELD HardSet mounting medium with DAPI. Detected green fluorescence is referred to autofluorescence of ergot alkaloids.

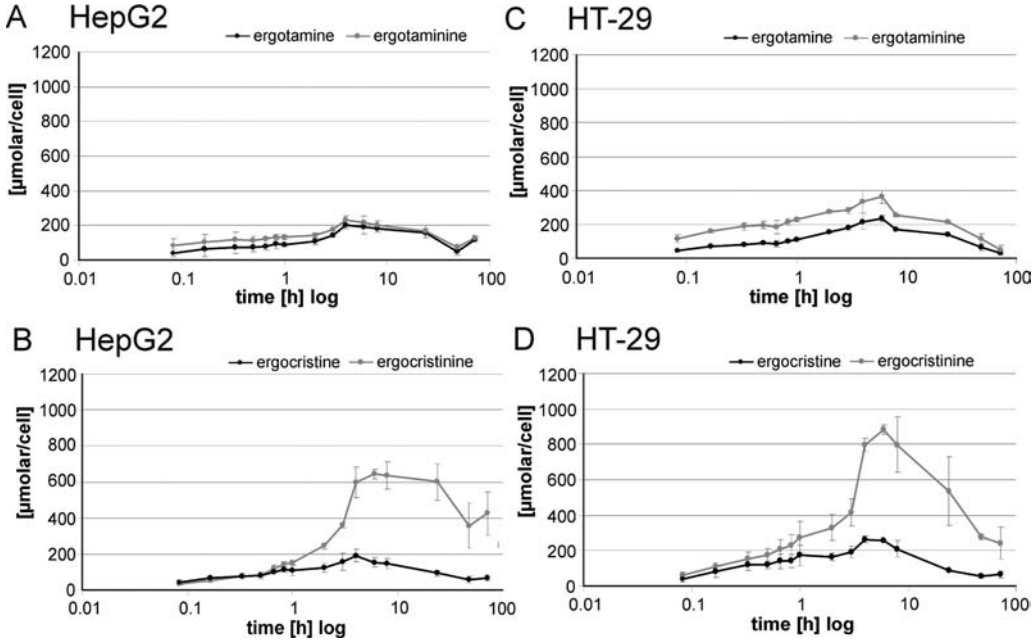

Figure 6. Time-dependent accumulation of ergotamine/ergotaminine (1 μM) and ergocristine/ergocristinine (1 μM) for (A and B) HepG2 and (C and D) HT-29. The cellular concentration (μM/cell) was corrected by the cell number and cell volume, measured with the CASY TT system. Number of analyzed samples (n) = 6.

dx.doi.org/10.1021/jf304569q | J. Agric. Food Chem. 2013, 61, 462−471

stained with VECTASHIELD HardSet mounting medium with DAPI to recover blue fluorescence. As displayed in Figure 7A, control cells only showed a blue fluorescence signal for cell nuclei. In contrast to this, ergot-alkaloid-incubated samples showed an additional bright green fluorescence around the cell nuclei. The fluorescence signal was more pronounced for 5 μM ergotamine/-inine (Figure 7B) than for cells treated with 1 μM ergocristine/-inine (Figure 7C).

DISCUSSION

Cytotoxicity. The results shown here represent a continuation of our previous studies on cytotoxic effects of ergot alkaloids in human primary cells.[21] In this context, it was interesting to analyze the toxic effects of ergot alkaloids in cell lines and compare these results to the effects observed in human primary cells. Experiments comparing primary cells and cell lines were described in the literature for other toxins, with significant differences.[30] It was a main aim of this study to identify the toxic impact of the single ergot alkaloids and perform monitoring to elucidate the most biologically active alkaloids.

The HT-29 and HepG2 cell lines were chosen for the following reasons: The metabolism of ergot alkaloids mainly takes place in the liver with excretion through bile.[32,33] Therefore, liver cells could be a main target for toxic effects, especially after accumulation of different ergot alkaloids. HT-29 cells also showed α-adrenergic receptor activity,[34] which make them possible targets for ergot alkaloids, because of the known receptor interaction.[18] Both cell lines represent cancer cells, a target known in the literature for synthetic ergot alkaloids, such as bromocriptine in tumor therapy.[25,35,36] In agreement with this anticancer activity, our results have shown a more pronounced effect of ergot alkaloids in the investigated cell lines compared to primary cells.[21] Peptide ergot alkaloids seem to have a greater impact on cells *in vitro* than lysergic acid amides, such as ergometrine. Data concerning cytotoxicity have shown very distinct effects of the six ergot alkaloids, as indicated in the literature.[4,37] We were able to calculate EC_{50} values for general cytotoxic effects using the CCK-8 assay (Table 1). It was clearly shown that, independent from the cell type, ergocornine, α-ergocryptine, and ergocristine have a high cytotoxic potential. In earlier studies, these three alkaloids were summarized in the ergotoxin group, which was later characterized as a composition of different substances.[37] Furthermore, they share a typical amino acid sequence, with L-valine replacing L-alanine (such as for ergotamine and ergosine) in the peptide moiety, as presented in Figure 1. Among the three mentioned alkaloids of the former ergotoxin group, toxic effects detected in the CCK-8 assay slightly differ, with ergocristine being the most toxic compound, followed by the other two. Less toxic effects were determined after incubation with ergosine or ergotamine (sharing L-alanine in the peptide moiety; Figure 1), depending upon the investigated cell line and ergometrine, as a lysergic acid amide with no cytotoxicity at all.

Further characterization of the most toxic ergot alkaloid was carried out in several other experiments. The observed effects were clearly characterized as apoptosis in both cell lines (Figures 4 and 5). Caspase-3 activation as a typical marker for apoptosis was detected in a high range, comparable to T-2 toxin, which is known to induce apoptosis.[30,31] For HepG2 cells, the three mentioned ergotoxin alkaloids, ergocornine, α-ergocryptine, and ergocristine, all incubated as an equilibrium mixture with their corresponding -inine form, showed a very high activation with over 1000% (20 μM) in comparison to control cells. These also natural occurring mixtures of both isomeric forms were necessarily used for all of the experiments because of a rapid conversion into each other. The applied concentration furthermore appeared to induce maximum effects, because caspase-3 activation values did not change for 10 and 20 μM α-ergocryptine and ergocristine. In contrast to this, the effect doubled for ergocornine within this concentration range, indicating a less cytotoxic effect of ergocornine compared to the other two ergotoxin alkaloids. All investigated cytotoxicity parameters of these alkaloids seem to be independent from the used cell type. In HT-29 cells, α-ergocryptine also showed high caspase-3 activation (1000%) and ergocristine-incubated samples resulted in an activation, comparable to T-2 toxin (Figure 4).

The results are in agreement with LDH release measurements (Figure 3). Because of the very high signals in the caspase-3 assay, a secondary necrosis *in vitro* of the substance could explain the observed LDH release.[38] All of our data have shown the same toxic properties of all six tested ergot alkaloids in both assays independent from the used cell line (LDH release and caspase-3 activation). When these data are taken into account, a high necrotic effect was detectable after 24 h of incubation for ergocristine, postulated as the most toxic alkaloid. A decrease in the caspase-3 signal is in consequence an effect of the strong necrosis induced by ergot alkaloids. These data are in agreement with lowered protein values for ergocristine-incubated samples (data not shown).

The caspase-3 activation results are in agreement with results from cell cycle analysis and confirm the apoptotic effect of the investigated ergot alkaloids. As shown in Figure 5, ergocristine-incubated samples showed a very high formation of the subG1 region, exceeding the effect of T-2 toxin and the other incubated alkaloids in HT-29 cells. Consequently, the data emphasized a stronger impact of ergocristine, being the most toxic peptide ergot alkaloid, already reflected by an EC_{50} value of about 1.5 μM (CCK-8 assay) in both cell lines. The data for the other ergot alkaloids concerning a subG1 formation also confirmed apoptosis induced by the peptide ergot alkaloids, although HepG2 cells appeared to be much less sensitive than HT-29 cells (Table 2). The apoptotic effect is in agreement with the description of chemically derived alkaloids, such as bromocriptine, inducing apoptosis in tumor cells,[22,23] as well as with our previous data showing apoptotic activity in primary cells.[21] Overall, when the investigations with both cancer cell lines are compared, HT-29 cells seemed to be a very suitable and sensitive model cell line to study the effect of ergot alkaloids.

When these data are taken together, the obtained sequence in terms of toxicological properties of the tested ergot alkaloids is ergocristine > α-ergocryptine > ergocornine > ergotamine and ergosine > ergometrine.

It is interesting to point out that this ranking represents an inversion of the elution of the compounds measured with reversed-phase C18 columns and, therefore, giving the hint that increasing hydrophobic properties results in enhanced cytotoxic effects.

Accumulation of Ergot Alkaloids in Cancer Cell Lines. To evaluate accumulation properties, ergotamine, ergocristine, and ergometrine (all in equilibrium with the corresponding -inine form) were chosen for accumulation experiments. Because of the fact that only small differences of ergocristine

and ergotamine occurred in their structure (replacing L-alanine for L-valine in biosynthesis; Figure 1) but the toxic property clearly differs, these were good examples as model substances. Additionally, the alkaloid composition in sclerotia is described to consist of 58% ergotamine and ergocristine, along with their corresponding -inine [8-(*S*) isomers] forms, making them quantitatively the most important ones.[14,17] The third substance ergometrine was chosen because of no detectable toxic effect in all of our measurements and because the structure as lysergic acid amide differs from the peptide alkaloids. Our results show a good correlation between postulated substance uptake/accumulation and cytotoxic properties because the most toxic compound ergocristine/-inine was accumulated in the highest range (panels B and D of Figure 6). It is important to highlight the evidence of substance concentrations not reaching zero over the time period of 72 h. The cells were not able to excrete the substance completely. Concentration curves measured in cell lysate are very similar to *in vivo* studies measuring free ergot alkaloid levels in plasma samples. It is reported, that the alkaloid concentration reaches a peak after only 1 or 2 h, as also reflected in our experiments.[39] Nevertheless, these *in vivo* experiments did not analyze an accumulation because only free alkaloids were measured in plasma samples. Consequently, these data are difficult to compare to each other. However, with our obtained data, it has to be questioned which isomeric form was detected in these experiments. Many (mostly older) data do not take the isomerization of the ergot alkaloids into account, referring to only one pure substance. Using a HPLC−FLD system for quantification, a distinct investigation of the two isomeric forms can be performed. Further experiments should focus on the differentiation of both isomeric forms because our recent experiments indicated different biological activities. To prevent any post-incubation isomerization in our study, the obtained cell lysates were immediately diluted in acetonitrile and stored under −20 °C.

As presented in our experiments, accumulation of the corresponding -inine form seems to be more pronounced than the corresponding -ine form, as shown for ergocristinine reaching up to 900-fold of the initial concentration (Figure 6D). In contrast to this, ergotaminine was enriched with 400-fold at the maximum, which is still a very high accumulation but in a significantly lower range compared to ergocristinine. With the use of the natural fluorescence properties, we were also able to visualize the accumulated ergot alkaloids in HT-29 cells. As shown in Figure 7, a green fluorescence could clearly be detected in ergot-alkaloid-incubated samples and not in equivalent control samples. A distribution over the whole cells could be observed with no characteristic enrichment in one part. For the first time, an ergot alkaloid accumulation was shown in cells without the use of any antibodies (Figure 7). This straightforward approach could be useful in future experiments, where the natural fluorescence of ergot alkaloids, combined with specific staining methods, may offer opportunities to elucidate transport mechanisms and binding to cellular components.

Several literature data suggest the formation of a toxin depot of ergot alkaloids and, consequently, an accumulation effect.[21,40−42] Binding of ergot alkaloids to cellular compartments cannot be excluded, inducing the described toxic effects. Taken the accumulation properties into account, even small concentrations could be enriched, having ultimately toxic properties. In contrast to all other tested compounds, ergometrine remains nontoxic in all assays and was not detectable in cell lysates. This fact was also shown in other cell types,[21] although receptor interaction properties have shown an impact of ergometrine.[20,43] Analogous to this, peptide ergot alkaloids, such as α-ergocryptine or ergotamine and ergocristine, have shown several effects in terms of receptor interactions, which were comparable to each other. These data differ from the cytotoxicity results in our study, obtaining large differences for individual ergot alkaloids. As already presented for primary cells, ergotamine/-inine and ergocristine/-inine mixtures differ significantly in their toxic properties.[21] These findings are comparable to our cytotoxicity results in cancer cell lines. When the accumulation data are taken into account, the concentration values for both -ine forms are comparable to each other (200-fold). A possible explanation for the difference in cytotoxic properties of the individual ergot alkaloids could be the enrichment of the 8-(*S*) isomers, characterized thus far as biologically less active. The corresponding -inine forms were accumulated in a much higher range than the 8-(*R*) isomers, and therefore, the effect could be explained by an effect of the 8-(*S*) isomers, enhancing cytotoxic properties of individual ergot alkaloids. Although the 8-(*S*) isomers are described to be biologically not or only weakly active,[11] the indications of possible other effects than receptor interaction are supported by the results of the current and previous study, although the concentration range of cytotoxic effects are in a much higher range compared to receptor interactions.[21] Recently published results also refer to an interaction of the 8-(*S*) isomers with the blood−brain barrier, which indicates the necessity for further research concerning ergot alkaloids.[44]

Besides accumulation properties, several metabolites were also detectable in our previous study.[45] Although HepG2 cells are derived from liver, the metabolic activity of them is discussed as very low in contrast to fresh isolated cells.[46,47] This is in agreement with our results. Hydroxy metabolites of peptide ergot alkaloids were detectable using HT-29 cells, but only traces were found in HepG2 cells.[45] These metabolites are also formed by mouse liver microsomes.[48] Furthermore, the hydroxy metabolites are described as receptor-active compounds, which may also lead to further effects in cells.[49] Because of the fact that the cytotoxic effects are higher in metabolically more active HT-29 cells and that the hydroxy metabolites are also receptor-active, the metabolism is not considered as a detoxification step. In conclusion, future research should focus on the cytotoxic potential of single isomers as well as the metabolites of ergot alkaloids.

Our results demonstrated cytotoxic and apoptotic effects of ergot alkaloids in two human cancer cell lines (HepG2 and HT-29 cells). In this *in vitro* model using the two cell lines, the cytotoxic potency of ergot alkaloids differs in a wide range, with some alkaloids showing antitumor properties similar to their synthetic derivatives. The peptide ergot alkaloids ergocornine, ergocryptine, and ergocristine (formerly known as ergotoxin) induced the most toxic effects with high caspase-3 activation, leading to a visible disruption of HT-29 cells. Overall, the data in cell lines underline the toxic properties observed in an earlier study.[21] Besides their cytotoxic effects, accumulation of ergot alkaloids was measured and a positive correlation between accumulation and cytotoxicity was confirmed in various measurements. Taking advantage of the natural fluorescence of ergot alkaloids, their accumulation was visualized by fluorescence microscopy in human cells without the use of

any antibodies. This approach could be useful in future investigations concerning accumulation and binding of ergot alkaloids to cytosolic compartments.

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Funding

We thank the NRW Graduate School of Chemistry for financial support.

Notes

The authors declare no competing financial interest.

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