Cell death in HeLa cells upon imperatorin and cisplatin treatment

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Abstract: There is growing evidence that commonly applied chemotherapy regimens can be improved by introducing new, specific, active and low side-effect drugs, or by combining substances to obtain the required clinical effect. The aim of the present study was to investigate the effects of imperatorin and cisplatin, applied separately or in combination, on apoptosis, necrosis and autophagy induction in the human cervical carcinoma cell line (HeLa). Imperatorin appeared to be a potent autophagy inducer, rather than a necrotic or apoptotic one. In contrast, cisplatin induced mainly apoptosis and necrosis after 6 h and 24 h, while longer incubation resulted only in necrosis induction. When HeLa cells were incubated with both drugs, autophagy appeared most frequently, although to a smaller extent than that observed after imperatorin administered alone. At the molecular level, autophagy was correlated with the presence of the cleaved form of microtubule-associated protein 1 light chain LC3 — LC3II. It was also accompanied by the inhibition of heat shock proteins Hsp27 and Hsp72 expression. Our results indicate that imperatorin alone, or in combination with cisplatin, is mainly an autophagy inducer in HeLa cells. (Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 3, 381–391)

Key words: imperatorin, cisplatin, cell death, HeLa, Hsp

Introduction

Imperatorin (IMP, 8-isopentenyloxypsoralene) belongs to the furanocoumarin family. It is a natural compound occurring in lemon, lime oils and popular culinary herbs like parsnip, fennel and parsley [1]. Imperatorin shows potent pharmacological activity and has been studied for its anti-inflammatory and antitumor properties. It has shown an antiproliferative effect on several cancer cell lines [2]. Suppression of cell proliferation and induction of apoptosis have been suggested as possible mechanisms for its anticancer activity [3]. Imperatorin inhibits the expression of cyclin D1 and induces G0/G1 cell cycle arrest in HeLa cells followed by apoptosis [4]. It also affects reactive oxygen species (ROS) production, induces mitochondrial membrane depolarization, decreases the level of antiapoptotic Bcl-2 and Bcl-x, and increases the proapoptotic Bax expression. Imperatorin promotes release of cytochrome c and activation of caspase 3 before leading to apoptosis [5]. Significant pro-apoptotic potential of imperatorin has also been confirmed by in vivo studies with nude mice bearing human hepatoma HepG2 cells [37]. In contrast, some authors have observed a mutagenic effect of imperatorin and transformation of the mouse fibroblast cell line [6, 7].
Cisplatin (CP, cis-dichlorodiammine-platinum (II)) is an important chemotherapeutic agent widely used in clinical treatment against several malignancies like ovarian, cervical, testicular, bladder, lung, gastric, head and neck cancers. At the molecular level, it interacts with cellular proteins and lipids and forms DNA adducts causing cell cycle arrest in G2/M phase [8–10]. It can also disturb cytoskeleton organization [11]. It may induce apoptosis through caspase 3-dependent and -independent signaling pathways. It has been shown that alteration in caspase 3 expression and activity were associated with the development of cellular resistance to cisplatin [12, 13]. There are also several other factors responsible for such resistance. One of them is heat shock proteins (Hsps) overexpressed in nearly all classes of tumors [14–17]. These are molecular chaperones, controlling proper folding of newly synthesized polypeptides, refolding of misfolded proteins, and controlling translocation through cell membranes protecting against cell death [18–21]. Therefore, tumour cell resistance to apoptosis is thought to be closely associated with Hsps overexpression, which indicates poor prognosis. Up-regulation of small heat shock protein Hsp27 has been observed in multiple malignancies including squamous cell carcinoma, gastric carcinoma, ovarian carcinoma and acute myeloid leukemia. High expression of Hsp72 has been reported in high grade malignant tumors such as pancreatic cancer, colorectal carcinoma, breast cancer, endometrial cancer, osteosarcoma and renal cell tumors. Overexpression of both proteins has also been found in cisplatin resistant human ovarian and cervical tumor cells [38]. Therefore, it seems that decreasing the Hsps level in cancer cells would be beneficial to patients.

Another type of programmed cell death observed in cancer cells is autophagy. This is caspase-independent cell death characterized by the accumulation of autophagic vacuoles in the cytoplasm accompanied by degradation of the Golgi apparatus and ER, preceding destruction of the nucleus. Autophagy is suppressed during the early stages of tumor progression. Therapeutically increased autophagy could represent an alternative way to destroy the cancer [22].

It is known that natural bioactive compounds may act in synergy with chemotherapeutics used in clinical treatment in programmed cell death induction. They also diminish the side effects of cytostatic drugs. Thus, the aim of our study was to investigate the effects of imperatorin and cisplatin, applied alone or in combination, on apoptosis, necrosis and autophagy induction in HeLa cells.

Material and methods

Cells and culture conditions. Human cervix carcinoma cell line (HeLa B, ECACC No 85060701) cultured in RPMI 1640 medium supplemented with 5% FBS (fetal bovine serum) (v/v) was used in this study. Cells were seeded on cover slides (for cell death detection) or in Falcon vessels (for heat shock proteins, caspase 3 and LC3 identification), at a density of 1 × 10^6 cells/ml and incubated at 37°C in humidified atmosphere with 5% CO₂.

Drug treatment. Imperatorin was isolated from the fruits of Angelica archangelica (Hofm) by column chromatography [39]. Imperatorin at final concentrations of 50 μM, 100 μM, 150 μM, and 200 μM, and cisplatin (Sigma) at final concentrations of 2 μM, and 5 μM were used in the experiments. The drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the culture medium did not exceed 0.01%, which, as indicated in preliminary experiments, did not influence cell viability or the expression of the proteins studied.

Three variants of drug treatment were performed:

1. The first variant — HeLa cells were incubated only with imperatorin or cisplatin for 6 h, 24 h and 48 h;
2. The second variant — imperatorin and cisplatin were added to the culture medium at the same time and incubated for 24 h;
3. The third variant — the cells were preincubated with imperatorin for 6 h followed by cisplatin treatment for the next 18 h; — the cells were preincubated with cisplatin for 6 h followed by imperatorin addition for the next 18 h. As a control, the cells were incubated with 0.01% of DMSO.

Immunoblotting. After imperatorin and/or cisplatin treatment, HeLa cells were lysed in a hot SDS-loading buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 10% glycerol; 100 mM DTT), boiled in a water bath for 10 min, centrifuged at 10,000 g for 10 min, and the supernatants were collected. The protein concentration was determined using the Bradford method [23], and samples of the supernatants containing 80 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis [24], and subsequently transferred onto Immobilon P membrane (Sigma). Following the transfer, the membrane was blocked with 3% low fat milk in PBS for 1 h, and incubated overnight with mouse monoclonal antibody anti-Hsp72 (SPA 810, StressGen) diluted 1:1,000, anti-Hsp27 (SPA 800, StressGen) diluted 1:1,000, and rabbit antibodies anti-caspase 3 active form (Sigma) diluted 1:500, anti-LC3 (Sigma) diluted 1:1,000. The membranes were washed 3 times for 10 min with PBS containing 0.05% Triton X-100 (Sigma) and incubated for 2 h with a 1:30,000 dilution of alkaline phosphatase-conjugated anti-mouse IgG.
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or anti-rabbit IgG (Sigma). The membranes were visualized with an alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a color development buffer (DMF, Sigma). The data was normalized relative to \( \beta\)-actin (Sigma, working dilution 1:2,000).

The level of protein expression was determined using Bio-Profil Bio-1D Windows Application V99.03 program. Three independent experiments were performed.

**Apoptosis detection with fluorochromes.** For apoptosis and necrosis identification, the cells were stained with fluorescent dye Hoechst 33342 (Sigma) and propidium iodide (Sigma), respectively [25]. Morphological analysis was performed under a fluorescence microscope (Nikon E — 800). Cells exhibiting blue fluorescent nuclei (fragmented and/or with condensed chromatin) were interpreted as apoptotic. Cells exhibiting pink fluorescent nuclei were interpreted as necrotic. At least 1,000 cells in randomly selected microscopic fields were counted under the microscope. Each experiment was performed in triplicate.

**Detection of acidic vesicular organelles with acridine orange.** Autophagy is a process of sequestrating cytoplasmic proteins into the lytic compartment and is characterized by the formation and promotion of acidic vesicular organelles (AVOs). To detect AVOs in HeLa cells treated with imperatorin and/or cisplatin, vital staining with acridine orange was performed [26]. The cells were incubated with the fluorochrome at a final concentration of 1 \( \mu \text{g/ml} \) for 15 min. Typical orange acridine — positive cells exhibited granular discretion of AVOs in the cytoplasm, indicative of autophagosome formation. Morphological analysis was performed under a fluorescence microscope (Nikon E — 800). At least 1,000 cells in randomly selected microscopic fields were counted under the microscope. Each experiment was performed in triplicate. The percentage of autophagic cells was calculated as the number of cells with AVOs versus the total number of stained cells.

**Combination index.** Determination of drug synergy or antagonism was quantified by the combination index (CI) [27]. CI provides a quantitative measure of the extent of drug interaction and the numerical value was calculated as described in this equation:

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CI = \frac{\text{Cisplatin} \% + \text{Imperatorin} \%}{\text{Cisplatin}/\text{Imperatorin} \%}
\]

Cisplatin \% and Imperatorin \% mean the percentage of dead cells after treatment with separate drugs, and Cisplatin/Imperatorin \% after treatment with both drugs. CI = 1 indicates an additive effect, < 1 — synergy, while > 1 — antagonism.

**Statistical analysis.** Data is presented as the mean of results ± SD coming from three independent experiments. Statistical evaluation was performed with one-way Anova test followed by Dunnet’s multiple comparison test. \( p < 0.05 \) compared to the control was taken to be the criterion of significance.

**Results**

**Effects of cisplatin and imperatorin on apoptosis, necrosis and autophagy**

For apoptosis, necrosis or autophagy estimation, control HeLa cells and cells treated with imperatorin and cisplatin separately, or in combination, were stained with Hoechst 33342, propidium iodide and acridine orange, respectively.

Imperatorin appeared to be an effective autophagy inducer rather than an apoptotic or necrotic one (Figure 1A). Our studies revealed a correlation between the drug concentration, incubation time and the number of autophagic cells. After 6 h of treatment, a not very high, but statistically significant, increase in the number of programmed dead cells was observed only after 100 \( \mu \text{M} \) of imperatorin. The highest percentage (18%) of autophagic cells was observed after 24 h of treatment with the 100 \( \mu \text{M} \) concentration of the drug. Higher concentrations were less effective, and a gradual decrease in the number of autophagic cells was observed. After treatment with 200 \( \mu \text{M} \), only about 6% of autophagic cells were found. After 48 h of treatment, a higher level of autophagic cells was observed after application of 150 \( \mu \text{M} \) of the drug (about 16%) and a dramatically decreased level when 200 \( \mu \text{M} \) was used.

In contrast to imperatorin, incubation of HeLa cells with cisplatin at the concentrations of 2 \( \mu \text{M} \) and 5 \( \mu \text{M} \) was less effective in autophagy induction (Figure 1B). The 24 h and 48 h incubations resulted in a significant increase in the number of apoptotic cells, especially after treatment with 5 \( \mu \text{M} \) of the drug (about 8%). After a longer incubation time, the apoptosis level was sustained, but it was accompanied by significant necrosis induction.

When HeLa cells were incubated with both drugs, autophagy was observed most frequently, but to a smaller extent than after treatment with imperatorin alone (Figure 2, Table 1). The intensity of the process depended on the drug concentrations and not significantly on the order of drug administration. Treatment with imperatorin at a concentration of 100 \( \mu \text{M} \) and cisplatin 2 \( \mu \text{M} \) was the most effective, and about 10–12% of autophagic cells were observed in each
incubation variant (Figures 2A–C). Co-incubation of HeLa cells with cisplatin 2 μM and imperatorin 150 μM at the same time was toxic to the cell culture and about 25% of necrotic cells were observed (Figure 2A). Incubation of HeLa cells with cisplatin 5 μM and imperatorin at different concentrations was less effective in autophagy induction than cisplatin 2 μM (Figures 2D–F). The highest percentage of autophagic cells was noted after treatment with 100 μM of imperatorin, especially when HeLa cells were preincubated with cisplatin. Incubation with higher imperatorin concentrations resulted in strong necrosis in all the drug combinations studied. Neither drug, in all the combinations studied, had a significant effect on apoptosis induction.

**Effects of imperatorin and cisplatin on Hsp72, Hsp27, caspase 3 and LC3 expression**

Apoptosis and autophagy at the molecular level are characterized by the expression of some marker proteins. Thus, we decided to estimate the level of caspase 3, LC3 as well as selected heat shock protein expression.

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**Figure 1.** The effects of different imperatorin (A) concentrations (0–200 μM) and cisplatin (B) concentrations (0–5 μM) on the levels of apoptosis, necrosis and autophagy induction in HeLa cells after 6 h, 24 h and 48 h of treatment. C — picture of autophagic cells stained with acridine orange after 24 h incubation with imperatorin (100 μM), D — picture of apoptotic and necrotic cells stained with Hoechst 33342 and propidium iodide after 24 h simultaneous incubation with cisplatin (5 μM) and imperatorin (150 μM), E — picture of apoptotic and necrotic cells stained with Hoechst 33342 and propidium iodide after 24 h incubation with cisplatin (5 μM). *p < 0.05
Heat shock proteins

The effects of imperatorin and cisplatin on the expression of Hsp27 and Hsp72 were drug- and concentration-dependent (Figures 3, 4). As shown in Figure 3, both drugs added to the culture medium, separately or in combination, generally were good inhibitors of Hsp27. Imperatorin was most potent after 24 h of treatment at the concentration of 100 μM (about 50%). Cisplatin diminished the protein level less effectively and the greatest inhibition was observed after 24 h and 48 h of incubation (about 80%). In the case of the drug combinations, simultaneous treatment of HeLa cells with imperatorin 150 μM and cisplatin 5 μM resulted in strong (about 20%) inhibition of Hsp27. Overexpression was observed only in one case — when HeLa cells were incubated with imperatorin at the concentration of 50 μM for 24 h (160%). In the case of Hsp72, imperatorin inhibited the protein level only after 48 h of incubation with 150 μM and 200 μM (about 25%), while cisplatin had no significant effect on the protein expression. A combination of both drugs, significantly or not, generally inhibited Hsp72 expression in all the variants studied. Overexpression was observed after 24 h of incubation with 50 μM and 200 μM of imperatorin, and after simultaneous treatment with cisplatin 5 μM and imperatorin 50 μM.

Caspase 3 and LC3

The effect of imperatorin on the expression of pro-apoptotic protein caspase 3 was time- and concentration-dependent (Figure 5A). After 6 h of treatment, no impact of the drug on the protein expression was observed, but longer incubation time (24 h) strongly increased its level, with maximum effectiveness at 50 μM (about 300%). Treatment with 200 μM of imperatorin had no effect on the protein expression. Increased protein expression (about 150%) was observed only after incubation with imperatorin 100 μM, but higher concentrations diminished the protein level.

Table 1. Combination index (CI) analysis of combined treatment of HeLa with imperatorin (IMP) and cisplatin (CP) for 24 hours. A — treatment with both drugs at the same time; B — cisplatin pretreatment followed by incubation with imperatorin; C — imperatorin pretreatment followed by cisplatin incubation; CI > 1 antagonism; CI < 1 synergism; CI = 1 additive effect

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imperatorin 50 μM, the level of caspase 3 was inhibited at all the variants of drug treatment (Figure 5D). Inhibition was also observed when the cells were preincubated with cisplatin followed by imperatorin treatment at the concentrations of 100 μM and 150 μM. When the cells were preincubated with imperatorin at concentrations exceeding 50 μM, the level of caspase 3 reached the control one. Overexpression of the protein was observed after simultaneous treatment of HeLa cells with 100 μM and 150 μM of imperatorin and 5 μM of cisplatin.

In the case of autophagy marker protein LC3, its cleavage into two forms, LC3I and LC3II, was observed after treatment with all the drug concentrations used in all experimental variants (Figure 6).

**Discussion**

There are conflicting reports concerning the effect of imperatorin on cancer cells. On the one hand, it has been indicated that imperatorin may be mutagenic, on the other hand, that it may inhibit mutagenesis...
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and cancerogenesis [6, 7]. Induction of programmed cell death, like apoptosis and autophagy, is regarded as an important factor of cancerogenesis action. Thus, we decided to examine the effect of imperatorin on apoptosis, necrosis and autophagy in HeLa cells. Our results indicate that imperatorin was a potent autophagy inducer, especially at the concentration of 100 μM after 24 hours of treatment and 150 μM after 48 h. A shorter incubation time was less effective. In contrast, cisplatin itself did not induce autophagy. At the molecular level, autophagy induction was correlated with specific expression of marker protein LC3. It is known that the cleavage of LC3 into two forms, LC3I and LC3II, is the hallmark of autophagy. LC3II participates in autophagosome formation and is associated with its structure. Accordingly, autophagy is associated with an increased ratio of LC3II to LC3I [28].

Several articles have appeared suggesting that induction of autophagy may block apoptosis [29]. In our experiments, imperatorin was a weak apoptosis inducer. Interestingly, it was correlated with strong caspase 3 expression, especially after 24 h and 48 h of treatment. It has been shown by other authors that imperatorin isolated from Cnidii monnieri fructus caused formation of apoptotic bodies, DNA fragmentation and enhanced degradation in HL-60 cells [30]. This was accompanied by activation of the cytochrome c-dependent pathway, mitochondrial membrane potential perturbation, release of cytochrome c, and in consequence, activation of caspase 3 and 9 [31]. Cisplatin induced apoptosis significantly only after 5 μM. It is known that cisplatin can induce apoptosis in caspase 3-dependent and -independent manners [12, 13]. In our experiments, the activation of caspase 3 was not significant. The mechanism involved in resistance of tumor cells to cisplatin is not clearly understood. It is supposed that the induction of resistance may be associated with decreased cellular cisplatin accumulation, enhanced DNA repair and increased glutathione (GSH) or

Figure 3. The effects of imperatorin (I) and cisplatin (C) on the level of Hsp27 expression. A — HeLa cells incubated with imperatorin (0–200 μM), B — cells incubated with cisplatin (0–5 μM), C — cells incubated with different imperatorin concentrations and cisplatin 2 μM, D — cells incubated with different imperatorin concentrations and cisplatin 5 μM; C+I — incubation with both drugs at the same time, IC — preincubation with imperatorin, followed by cisplatin treatment, CI — preincubation with cisplatin, followed by imperatorin treatment. *p < 0.05
increased Hsps levels [32, 33]. In our experiments, cisplatin had no significant impact on Hsp72 expression and inhibited Hsp27 at the concentration of 5 μM after 24 h and 48 h of treatment. At these concentrations, apoptosis as well as necrosis was observed, which may suggest the involvement of Hsp27 in cisplatin resistance of HeLa cells to death.

Many studies have demonstrated that natural plant extracts and natural compounds exhibit a synergistic anti-tumor effect with chemotherapeutics [34, 35]. Thus, we decided to examine the effect of imperatorin on the cisplatin-induced apoptosis of HeLa cells. We measured cell death in HeLa cells pretreated with imperatorin or cisplatin followed by cisplatin or imperatorin treatment and compared the effect to cell death induced by cisplatin and imperatorin used simultaneously. In our experiments, the combination of both drugs was most effective in autophagy induction, especially when imperatorin at the concentration of 100 μM was present in the drug mixture. Similar results were observed after imperatorin itself. Surprisingly, the effectiveness of autophagy induction was smaller in the case of the combination than in the case of imperatorin alone. This may suggest that cisplatin diminishes the pro-autophagal properties of imperatorin. Autophagy induction was correlated with the increased level of cleaved form LC3II. Inhibition of Hsp72 and Hsp27 was also observed.

Neither drug at all the combinations had a significant effect on apoptosis induction. Only after simultaneous treatment of imperatorin over 100 μM with cisplatin 5 μM was an increased level of apoptotic cells observed. It was correlated with an increased level of caspase 3. Unfortunately, this was also accompanied by strong necrosis induction, suggesting a toxic effect of both drugs on the cell line. Our earlier results strongly suggested that the significant decrease in Hsp72 expression after pretreatment with a natural flavonoid, quercetin, was correlated with the highest sensitivity of HeLa cells to cispl-
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Figure 5. The effects of imperatorin (I) and cisplatin (C) on the level of caspase 3 expression. A — HeLa cells incubated with imperatorin (0–200 μM), B — cells incubated with cisplatin (0–5 μM), C — cells incubated with different imperatorin concentrations and cisplatin 2 μM, D — cells incubated with different imperatorin concentrations and cisplatin 5 μM; C+I — incubation with both drugs at the same time, IC — preincubation with imperatorin, followed by cisplatin treatment, CI — preincubation with cisplatin, followed by imperatorin treatment. *p < 0.05

atin-induced apoptosis [36]. In the present study, the inhibition of Hsp72 as well as Hsp27 by imperatorin was correlated with increased sensitivity to autophagy rather than apoptosis.

In summary, our results indicated that imperatorin alone, or in combination with cisplatin, was very effective in inducing autophagy in HeLa cells. The pro-autophagic activity of imperatorin was mediated by LC3 cleavage and the inhibition of heat shock proteins expression, especially Hsp27.

References
**Figure 6.** The effects of imperatorin (I) and cisplatin (C) on the level of LC3 expression. A — HeLa cells incubated with imperatorin (0–200 μM), B — cells incubated with cisplatin (0–5 μM), C — cells incubated with different imperatorin concentrations and cisplatin 2 μM, D — cells incubated with different imperatorin concentrations and cisplatin 5 μM; C+I — incubation with both drugs at the same time, IC — preincubation with imperatorin, followed by cisplatin treatment, CI — preincubation with cisplatin, followed by imperatorin treatment. *p < 0.05


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