Combined effect of sodium selenite and campthotecin on cervical carcinoma cells*

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The effects of selenite, campthotecin and their combination were investigated in cervical carcinoma cell line Hep-2 HeLa during 24h. The measured parameters included morphological changes, proliferation, oxidative stress, mitochondrial status, caspase-3 activation and nuclear fragmentation. Selenite at all but lowest concentrations inhibited cell growth and proliferation and induced cell death characterized by membrane blebbing, oxidative stress and mitochondrial damage, occurring in the absence of caspase-3 activation and nuclear fragmentation. Campthotecin at all concentrations induced gradual apoptosis including all measured morphological and molecular parameters with exception of oxidative stress. A combination of selenite and campthotecin induced both antagonistic and synergistic effects on cervical carcinoma cells. While low selenium concentration slightly reduced cytotoxicity and proapoptotic effects of campthotecin, moderate and higher concentrations of selenium enhanced them, changing simultaneously apoptosis into more necrosis-like death. These results show importance of selenium as a potential modulator and enhancer of campthotecin-based anticancer therapy in nonovarian malignancies.

Key words: selenite, campthotecin, cervical cancer, apoptosis, mitochondria

Selenium is an essential microelement whose putative antineoplastic properties have attracted considerable interest over past years. Many experimental studies have demonstrated that selenium is capable of protecting cells and tissues against various types of chemical and biological carcinogens both *in vitro* and *in vivo* [1, 10, 12, 28, 31]. Furthermore, several epidemiological studies have shown a relationship between low plasma selenium content and tumor incidence in humans [5, 7, 12, 16].

The exact mechanism(s) whereby selenium exerts its chemopreventive effects is still difficult to pinpoint as this element appears to play the dichotomous role in the living organism. On the one hand, selenium protects against oxidative stress by being a part of glutathione peroxidase (GP_X), which scavenges free radicals responsible for oxidative damage of DNA and other macromolecules [20, 27, 32]. On the other hand, upon higher concentrations selenium may function as a prooxidant, catalyzing the oxidation of thiols and generating superoxide anion [4, 26, 33]. In addition, selenoglutahione, a metabolic product of selenite, can specifically inhibit protein synthesis and activate p53 mediated apoptosis in tumor cells [17].

Campthotecin (CPT) and its derivatives are topoisomerase I inhibitors which have been shown to be potent anticancer agents, inducing apoptosis by promoting double strand DNA breaks during S phase of the cell cycle [11, 21, 25]. Traditional targets for CPT-based chemotherapy include ovarian, lung, breast and colon cancer; however, recently it has been demonstrated that some CPTs have significant cytotoxic effects in cervical carcinoma cells too [3].

In this study, we examined the effects of sodium selenite, CPT and their combination on cell proliferation and cell death while focusing on the possible mechanisms of their

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effects. We chose Hep-2 HeLa cells, an epitheloid cervical cell line because selenium might play a role in pathogenesis of carcinoma of the uterine cervix and CPTs are among new cytotoxic agents which are being clinically studied in this disease [8].

Material and methods

Chemicals. Sodium selenite; CPT; cytochrome c; 4',6-diamidino-2-phenylindole (DAPI) and Triton-X were purchased from Sigma-Aldrich (Prague, Czech Republic). 4-[3-(4-Iodophenyl) -2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzen e disulphonate (WST-1) was from Boehringer Mannheim-Roche (Basel, Switzerland). Secondary antibodies for caspase-3 detection were from Molecular Probes, Inc. (Eugene, USA) and from EXBIO (Prague, Czech Republic). Monospecific antiserum for the detection of activated caspase-3 was obtained from New England Biolabs, Inc. (Beverly, USA). All other chemicals were of highest analytical grade.

Cell culture and treatment. The continuous cell line Hep-2 HeLa (ECACC, No. 86030501, Porton Down, Great Britain) was cultivated in a humidified 5% CO_2 atmosphere at 37 ˚C in Dulbecco's modified Eagle's medium (DMEM) (Sevapharma, Prague, Czech Republic) with 10% bovine serum (ZVOS Hustopeče, Uherčice, Czech Republic), penicillin G (100 U/ml) and streptomycin (100 μg/ml). For experiments, the cells were seeded into cultivation flasks (Nunclon, Roskilde, Denmark), modified cytospin chambers (Hettich, Tuttlingen, Germany) or 96-well plates (Nunclon, Roskilde, Denmark).

Stock solutions of sodium selenite and CPT were prepared in serum-free DMEM (selenite) or DMSO (CPT) and stored in a refrigerator until use. Before each experiment, stocks were diluted to the final concentration in DMEM with 10% BS. Experiments were repeated at least three times.

WST-1 cytotoxicity assay. Cells were seeded into 96-well plates at concentration of 6,000 cells/well, always with the first column of wells without cells (blank), and allowed to grow overnight. After 24 h, the medium was aspirated and cells were exposed to 200 μl/well of freshly prepared medium containing selenite, CPT or their combination for 24h. At the end of each experiment, the medium was removed; cells were washed twice and exposed to medium with 100 μl of WST-1. After 2 h, the changes in absorbance were recorded by a scanning multiwell spectrophotometer Titertec Multiscan MCC/340 (ICN Biochemicals, Frankfurt, Germany) at 450 nm with 650 nm of reference wavelength. In all cases the absorbance of the tested substance in medium alone was recorded to determine whether it interfered with the assay.

Morphology of cells. Hep-2 HeLa cells were grown in DMEM under standard laboratory conditions (37 ˚C, 5% CO_2). After 24 h cultivation, medium was replaced with a medium containing varying concentrations of selenite, CPT or their combination. Time-lapse videomicroscopy of treated cells vs. control cells was carried out in a 37 ˚C heated chamber, using the inverted microscope Olympus IX-70 (Olympus Optical Co, Ltd., Tokyo, Japan) equipped with the Mitsubishi CCD-100E camera (Mitsubishi Corporation, Tokyo, Japan) and connected to the Mitsubishi time-lapse video recorder HS-S5600 (Mitsubishi Corporation, Tokyo, Japan). The recording was performed in a 480 mode, with a slowing factor of 160 and it continued for 24 h, with a subsequent video analysis. All time-lapse sequences were converted to digitized form by the software Adobe Premiere 6.0 (Adobe Systems Incorporated, San Jose, USA) and analyzed. Simultaneously, digital photographs of experimental cultures were taken with the digital camera Olympus C-4040 (Olympus Optical Co, Ltd., Tokyo, Japan) at different time intervals.

Detection of apoptosis by caspase-3 activation and DAPI staining. Exposed and control cells in cytospin chambers were fixed with 1 ml of 4% formaldehyde for 1 h. The presence of the active form of caspase-3 was followed under the fluorescence microscope Nikon Eclipse E400 (Nikon Corporation, Kanagawa, Japan) using FITC and DAPI specific filters [24]. In all experiments, the simultaneous immunodetection of caspase-cleaved neo-epitome of cytokeratin 18 was performed to verify caspase-3 positivity. Furthermore, all the specimens were counterstained with DAPI to see apoptosis specific nuclear changes.

Mitochondrial potential analysis. Hep-2 HeLa cells were seeded into cytospin chambers and allowed to grow overnight under standard laboratory conditions (37 ˚C, 5% CO_2). Next day, cultures were treated with selenite, CPT or their combination at different concentrations during various time intervals. After each time interval, cultures were centrifuged for 5 minutes at 500 rpm (JOUAN, Nantes, France) and room temperature (RT). The medium was removed and changes in mitochondrial transmembrane potential were determined by MitoCapture^{TM} Mitochondrial Apoptosis Detection Kit (BioCat GMbh, Heidelberg, Germany). Specific signals were detected by fluorescence microscopy – Nikon Eclipse E400 (Nikon Corporation, Kanagawa, Japan) equipped with the digital color matrix camera COOL 1300 (VDS, Vosskühler, Germany), using band pas filter (detects FITC and rhodamine). The resulting shifts from red to green fluorescence were quantified by cell counting and analysis in at least 200 visual fields using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic).

Measurement of oxidative stress. Hep-2 HeLacells were seeded into 96-well plates at concentration of 6,000 cells/well, always with the first column of wells without cells (blank), and allowed to grow overnight. After 24 h, the

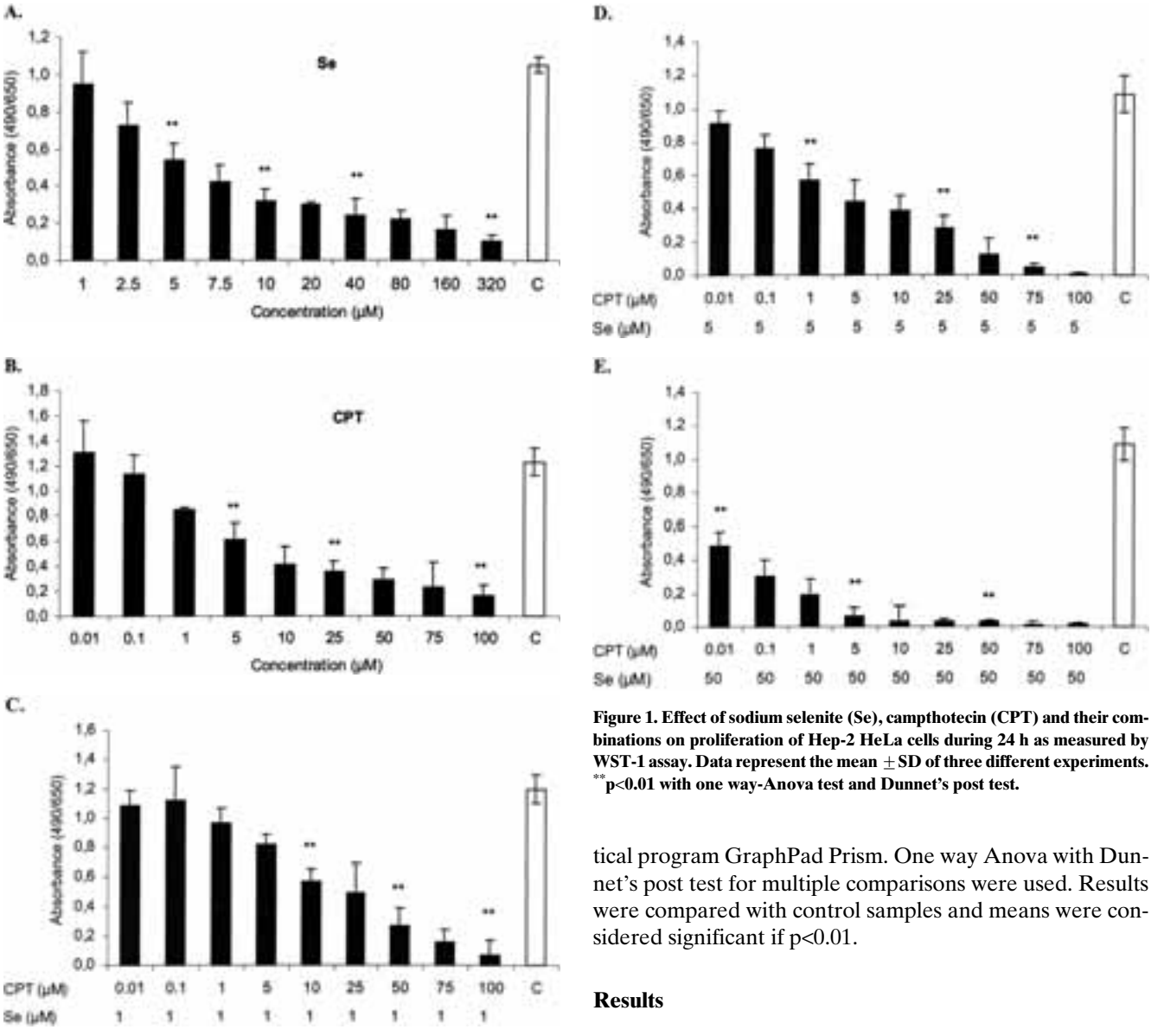

Figure 1. Effect of sodium selenite (Se), campthotecin (CPT) and their combinations on proliferation of Hep-2 HeLa cells during 24 h as measured by WST-1 assay. Data represent the mean ± SD of three different experiments. **p<0.01 with one way-Anova test and Dunnet's post test.

medium was aspirated and cells were exposed to 200 μl/well of freshly prepared medium containing selenite, CPT or their combination for different time intervals. At the end of each experiment, the medium was removed; cells were washed twice and incubated in 100 μM ferri-cytochrome c solution for 1 h. The absorbance at 550 nm was subsequently read with help of scanning multiwell spectrophotometer Titertec Multiscan MCC/340 (ICN Biochemicals, Frankfurt, Germany). Protein content was determined by bicinchoninic acid assay – BCA assay (Bicinchoninic acid kit for protein determination, Sigma-Aldrich, Prague, Czech Republic). The level of superoxide anion was calculated from the standard calibration curve produced by cytochrome c alone and expressed as nM/mg protein.

Statistics. Statistical analysis was carried out with a statistical program GraphPad Prism. One way Anova with Dunnet's post test for multiple comparisons were used. Results were compared with control samples and means were considered significant if $p<0.01$.

Results

Cytotoxicity. At 24 h treatment, both sodium selenite and CPT inhibited proliferation of Hep-2 HeLa cells in a concentration-dependent manner (Fig. 1A, 1B). To determine whether selenium enhances antiproliferative and cytotoxic effect of CPT, cells were exposed to a combination of both chemicals and the number of viable cells was measured. Figure 1 next shows that sodium selenite had both synergistic as well as antagonistic effect on cytotoxicity of CPT during 24 h. Selenite at a concentration of 1 μM slightly decreased cytotoxicity of CPT at all employed concentrations (Fig. 1C). On the other hand, selenite concentration of 5 μM and, in particular 50 μM had a significant additive effect on CPT cytotoxicity during 24 h (Fig. 1D, 1E).

Dynamic morphology of treated cells. Low concentra-

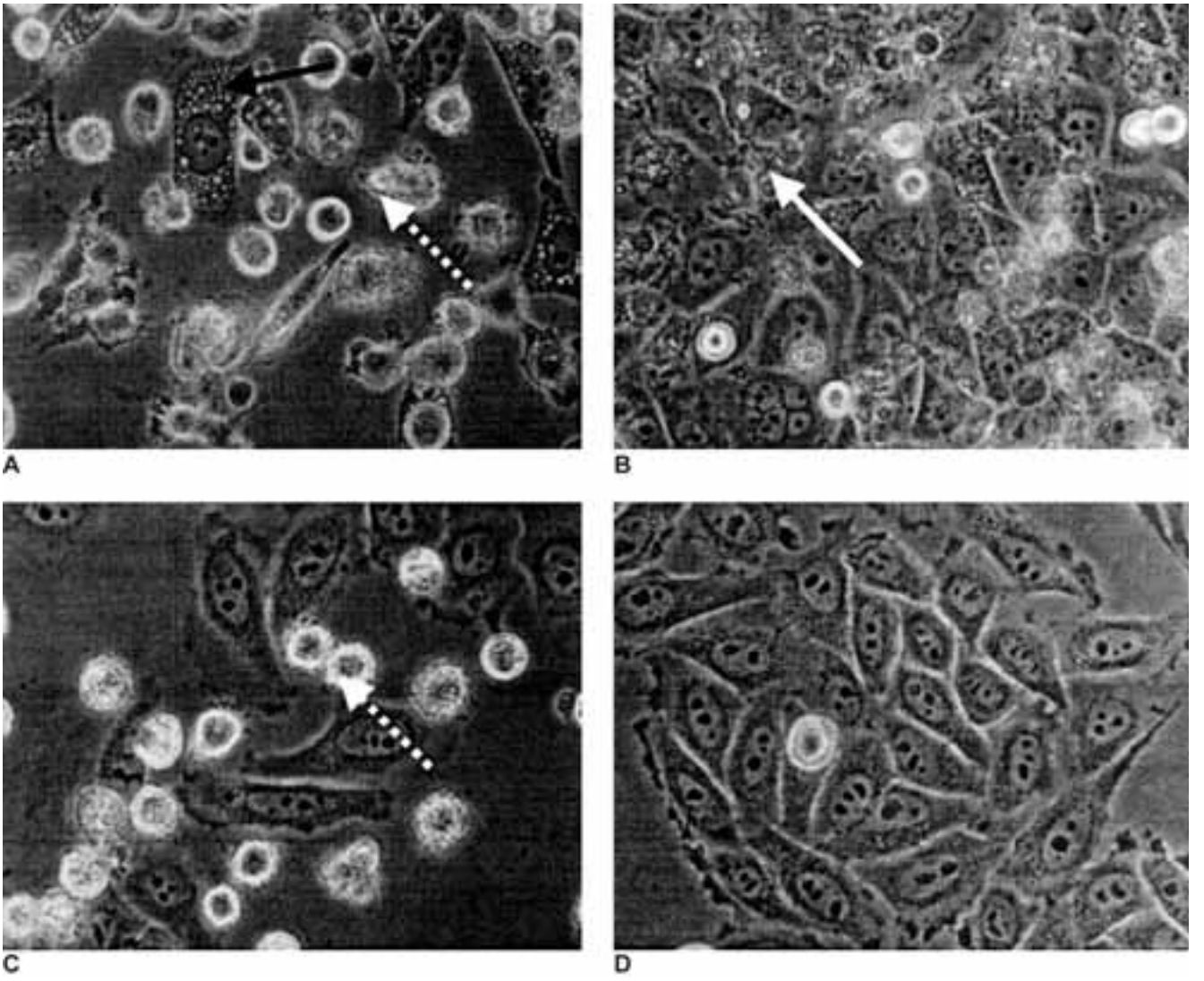

Figure 2. Morphology of Hep-2 HeLa cells after (A) 5 μM sodium selenite, (B) 50 μM sodium selenite, (C) 10 μM campthotecin; (D) untreated control culture. Black arrow denotes vacuolization in selenium-treated cells. White arrow shows necrosis-like dying cells in selenium-treated cells. White dashed arrows point at blebbing cells in selenium and campthotecin-treated cultures. Phase contrast 600x.

tions of both sodium selenite (5<μM) and CPT (1<μM) did not produce any observable effects in Hep-2 HeLa cells during 24h. Moderate concentrations of sodium selenite (5–20 μM) induced formation of vacuoles in the cytoplasm of exposed cells, cell rounding and shrinkage, followed by gradual loss of adherence and blebbing, which continued for varying time intervals (Fig. 2A). Upon higher concentrations (40–320 μM), selenium induced a very rapid degradation of cells which occurred during first hours of treatment. The cells lost adherence, shrinking rapidly in the absence of membrane blebbing (Fig. 2B).

The effect of moderate concentrations of CPT (1–25 μM) on Hep-2 HeLa cells was essentially the same as with selenium, with only difference being the absence of vacuoles in the cell cytoplasm (Fig. 2C). There was no qualitative difference in the appearance of dying cells after higher concentrations of CPT (25–100 μM); the entire process was just accelerated. The combined effect of selenium (5 and 50 μM) and CPT (1–25 μM) on Hep-2 HeLa cells during 24 h showed no qualitative difference in the appearance of cells in comparison with cultures treated with selenium and CPT alone (data not shown).

Selenium induces cell death bearing features of apoptosis and necrosis. To find out more about the nature of cell death and mechanisms by which selenium stimulates it, we selected several apoptotic markers; i.e. functional status of mitochondria, the presence of the active form of caspase-3 and nuclear fragmentation whose appearance coincides with different phases of this phenomenon. Moreover, since

one of the proposed mechanisms of selenium toxicity is generated oxidative stress; we measured amounts of superoxide anion in the treated cells.

Sodium selenite at low concentrations had no effect on the above mentioned markers (data not shown). At moderate concentrations, however, sodium selenite interfered with mitochondria in the exposed cells, causing an early decrease of mitochondrial transmembrane potential as seen on Figure 3A. On the other hand, other apoptotic markers – caspase-3 activation and chromatin fragmentation were not significantly increased in the exposed cells in comparison with control cultures at all followed intervals of the treatment (Fig. 4A and 5A). Also, sodium selenite at moderate concentrations generated oxidative stress in Hep-2 HeLa cells, the levels of superoxide anion detected in the treated cells were significantly higher as soon as 6 h after the beginning of treatment (Fig. 6A).

High concentrations of sodium selenite produced a severe damage to mitochondria measurable already after 5 min of treatment along with the absence of other apoptotic markers and oxidative stress (data not shown).

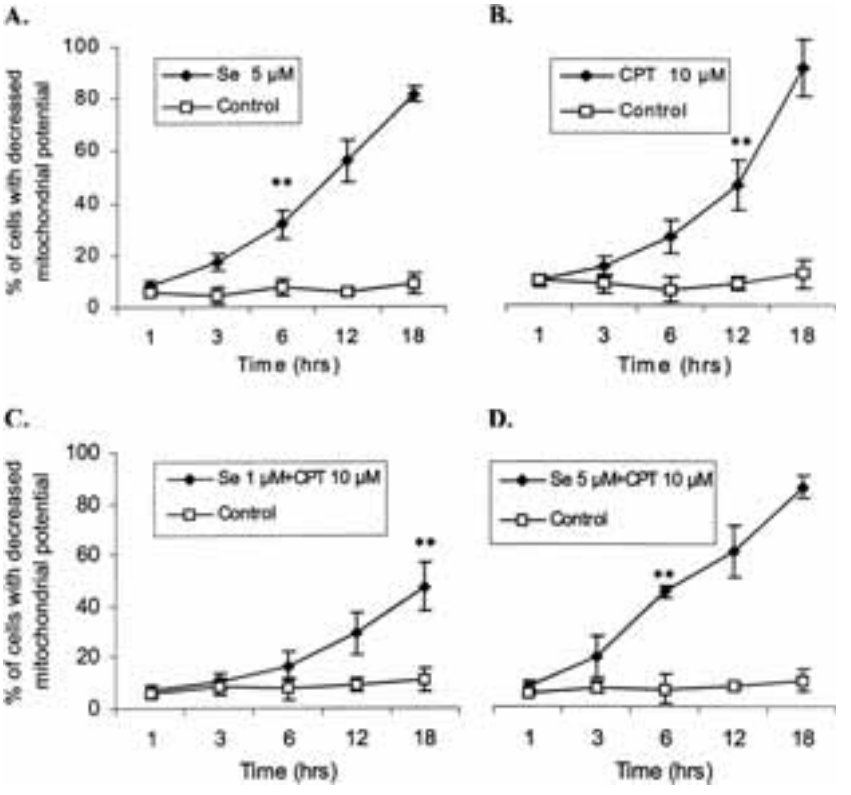

Figure 3. Changes in mitochondrial potential after (A) 5 μM sodium selenite (Se), (B) 10 μM campthotecin (CPT), (C) 1 μM Se + 10 μM CPT; (D) 5 μM Se + 10 μM CPT during 24 h. Data represent the mean $\pm$SD of three different experiments. **p<0.01 with one way-Anova test and Dunnet's post test.

CPT induces apoptosis with limited contribution of oxidative stress. CPT-induced apoptosis in Hep-2 HeLa cells was concentration and time-dependent, with optimal induction at CPT concentrations between 1 and 25 μM during 24 h. The process was characterized by the decreased mitochondrial potential, the presence of the active form of caspase-3 and specific nuclear fragmentation (Fig. 3B, 4B, 5B). Superoxide anion levels detected in the CPT-treated cells were slightly increased at 6 h of the treatment but at later time intervals a downtrend followed (Fig. 6B).

Selenium has ambiguous effects on CPT-induced apoptosis. Since selenite specifically influenced the cytotoxicity of CPT, we wanted to know whether this interaction affected the course and extent of apoptosis too. Selenite at the concentration of 1 μM delayed CPT-induced disruption of mitochondrial potential, caspase-3 activation, chromatin fragmentation and superoxide anion amounts (Fig. 3C, 4C, 5C, 6C). On the other hand, moderate and higher concentrations of selenite (5 and 50 μM) significantly enhanced cytotoxicity of the selenite-CPT combination, in particular toward mitochondria and increased oxidative stress (Fig. 3D, 6D). The detected levels of activated caspase-3 and chromatin fragmentation were found decreased at all employed treatment combinations in comparison with CPT alone (Fig. 4C, 5C).

Discussion

Currently, advanced and recurrent cancer of the uterine cervix is treated with a combination of radiotherapy and cisplatin. Still, this modality is often not sufficient, and thereby new treatment strategies and options are sought to improve a cure rate for patients with this and other nonovarian malignancies [8].

Recently, CPTs have been found to have cytotoxic effects in several carcinoma cell lines of the cervix. Furthemore, topotecan, a CPT derivative, showed promising antitumor activity in patients with advanced or recurrent cervical cancers. Therefore, new studies using CPTs alone or in combination are required and planned to fully characterize their potential in the treatment of nonovarian cancers [2, 14, 23].

Here, we first demonstrated that CPT at moderate concentrations (1–25 μM) is cytotoxic in Hep-2 HeLa cervial carcinoma cell line during 24 h (Fig. 1B). Dynamic morphology of CPT-treated cells as seen on digitalized sequences

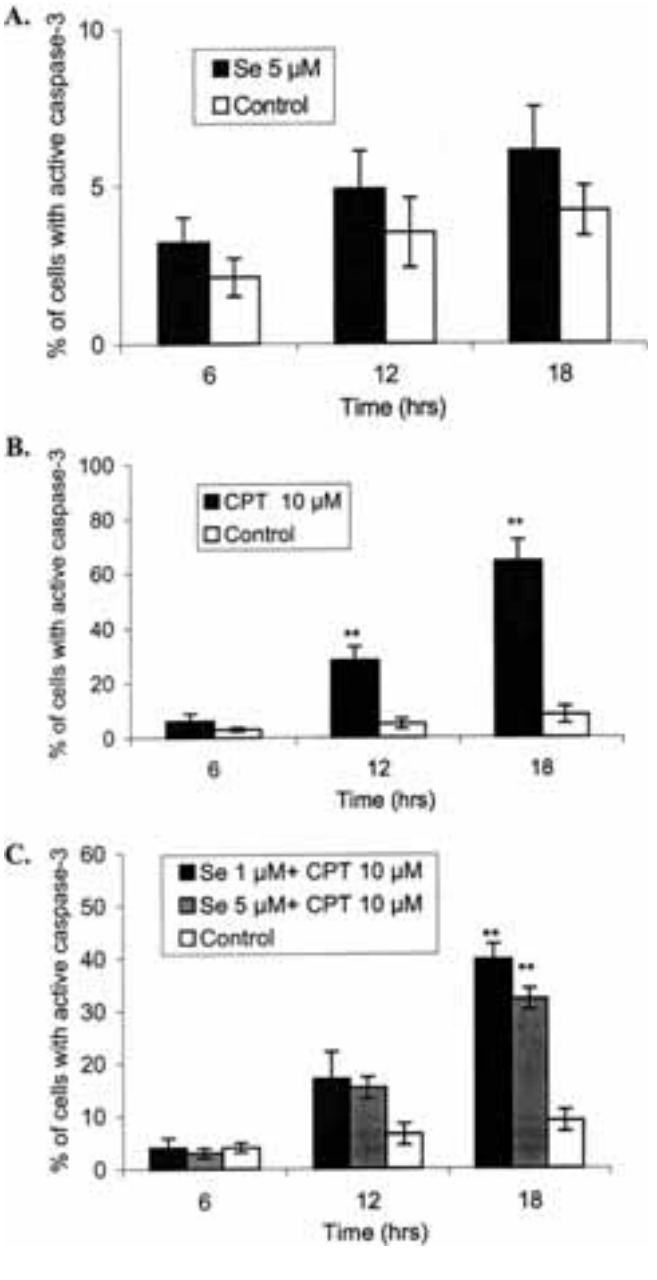

Figure 4. Time course of caspase-3 activation in Hep-2 HeLa cells treated with (A) 5 μM sodium selenite (Se), (B) 10 μM campthotecin (CPT), (C) 1 μM Se + 10 μM CPT and 5 μM Se + 10 μM CPT during 24 h. Data represent represent the mean $\pm$SD of three different experiments. **p<0.01 with one way-Anova test and Dunnet's post test.

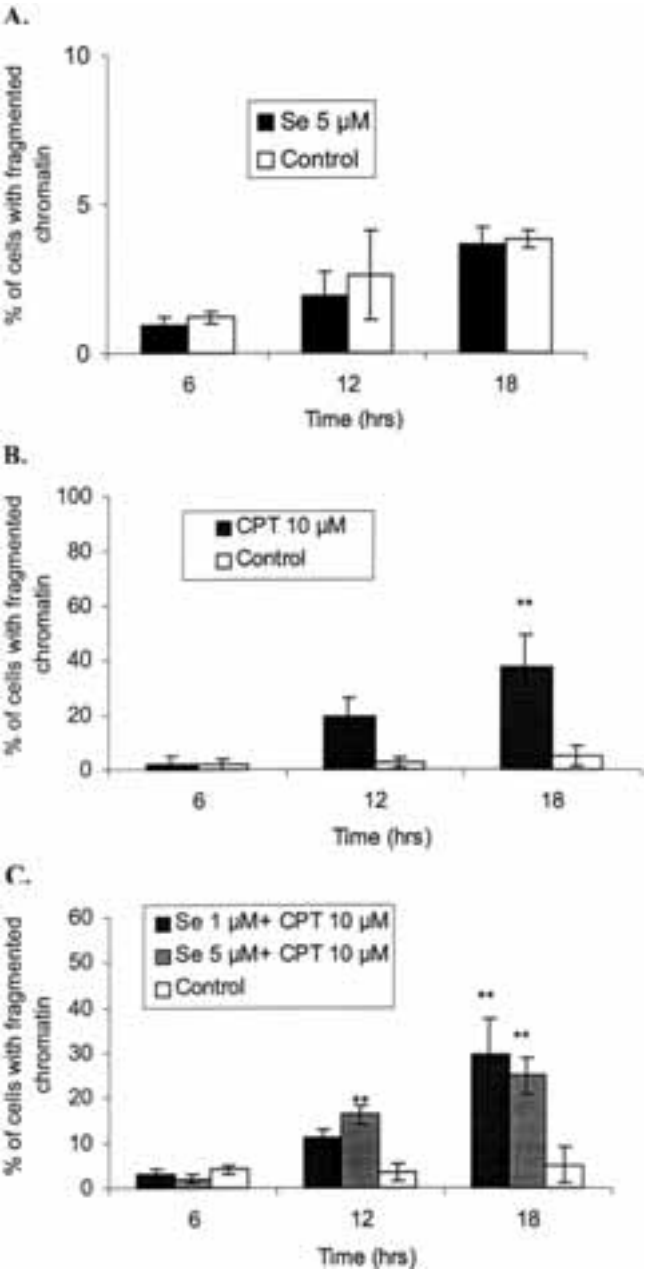

Figure 5. Chromatin fragmentation in Hep-2 HeLa cells treated with (A) 5 μM sodium selenite (Se), (B) 10 μM campthotecin (CPT), (C) 1 μM Se + 10 μM CPT and 5 μM Se + 10 μM CPT during 24 h. Values are the mean $\pm$SD of three different experiments. **p<0.01 with one way-Anova test and Dunnet's post test.

shows a gradual loss of adherence of individual cells followed by their shrinkage and violent blebbing which lasted approximately up to two hours. Although this process was not synchronized in the entire cell population, by 24 h, all cells underwent the blebbing stage and remained motionless, releasing their content through the damaged membrane. This observation corresponds to the known fact that CPTs act mostly on cells in S phase which in our case constituted about 10-20 percent of growing cell population (unpublished observations) and therefore CPT-related effects should be gradual. CPTs have been shown to induce apoptosis in different cell lines via DNA damaging, with subsequent activation of mitochondrial pathway by down-regulating Bcl-2 and promoting translocation of Bax and

subsequent release of cytochrome c [6, 15, 25]. Still, it appears that other mechanisms are involved in this process too, as CPT might interfere with MAPK signaling pathway and does not always activate selected caspases as has been demonstrated in human gastric cancer cell line AGS [18]. In this study, we have shown that CPT caused a decrease in mitochondrial potential and the activation of the executionary caspase-3, which was seconded by chromatin fragmentation, thus confirming that in Hep-2 HeLa cells this chemical induces apoptosis by activation of mitochondrial pathway.

Inorganic selenite has been demonstrated to protect cells against elicited oxidative stress as well as cause their damage, and both mechanisms are nowadays considered as possible underlying reasons for its antineoplastic potential [4, 10, 13]. Also, it has been observed that the effects of selenite are strongly concentration dependent, with lower doses being protective and higher doses destructive [19, 26]. In accord with these observations, moderate concentrations of sodium selenite induced oxidative stress which was linked to an alteration in mitochondrial potential and subsequent activation of apoptosis in our model (Fig. 6A). Nevertheless, this process was not associated with the activation of caspase-3 as well as fragmentation of chromatin. This discrepancy might be ascribed to several factors. Firstly, selenite exerted damage to mitochondria as well as to other molecules could have been too extensive and rapid to allow a rather orderly sequence of apoptotic steps and the entire process thus turned toward necrosis. This hypothesis is further supported by our observations of the extensive necrosis-like vacuolization during treatment of cells and by the detected levels of generated oxidative stress. Secondly, there seems to be a very fine threshold between chemically induced apoptosis and necrosis, depending on the employed dose of the particular chemical and the type of experimental model. Our treatment schemes were not primarily aimed at determination of this threshold – concentration relationship and therefore it could have escaped our attention. It is also tempting to speculate about a possibility of a new intermediate type of cell death which has already been reported in connection with chemical treatment too [9, 30].

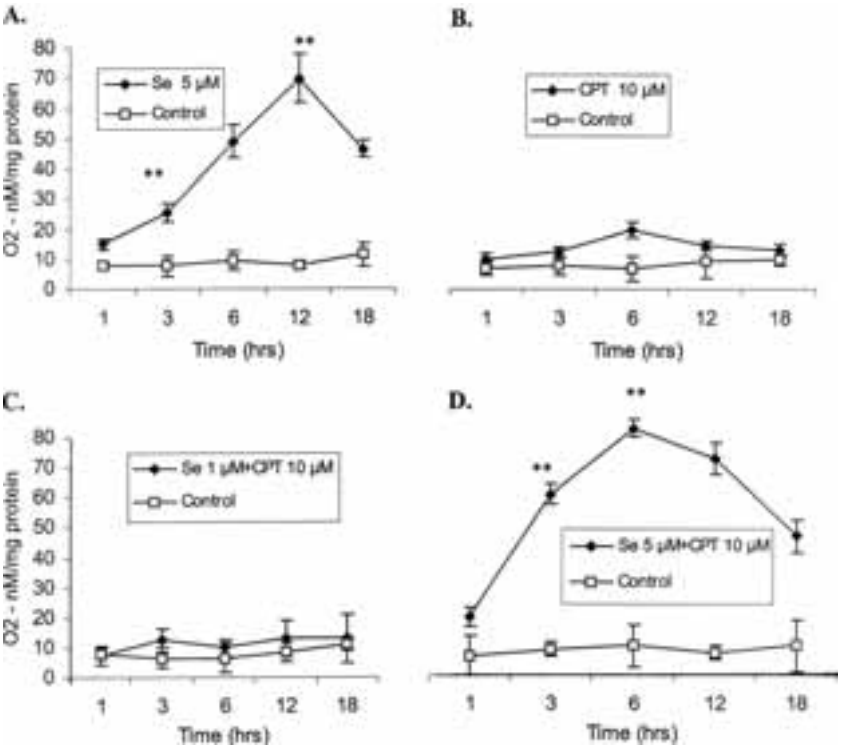

Figure 6. Superoxide anion production as an indication of oxidative stress in Hep-2 HeLa cells after (A) 5 μM sodium selenite (Se), (B) 10 μM campthotecin (CPT), (C) 1 μM Se + 10 μM CPT and 5 μM Se + 10 μM CPT during 24 h. Cells were treated as described above and the superoxide anion production was determined from oxidation of ferri-cytochrome c solution measured spectrophotometrically at 550 nm. Values are the mean $\pm$ SD of three different experiments. $^{**}p<0.01$ with one way-Anova test and Dunnet's post test.

We investigated the combined effect of selenium and CPT on Hep-2 HeLa cells during 24 h. Our hypothesis was that selenium whose activity is generally not cell cycle specific but may block the cell cycle at the S/G_2-M phase could potentiate S-phase specific proapoptotic effects of CPT [27]. We found that selenium had both synergistic as well as antagonistic effects on CPT-promoted cytotoxicity and cell death. Antagonism of selenium at 1 μM and CPT at low concentrations (Fig. 1C) seems to be relatively understandable in the light of already published papers. Many studies have demonstrated a protective role of selenium against DNA damaging chemicals and radiation [20, 22, 32]. This protection has been attributed to antioxidant properties of selenium-enhanced GP_X and other selenium containing enzymes [13, 16, 32]. On the other hand, oxidative stress detected in CPT-treated cells was not significantly increased to allow us suppose that this was the major mechanism of protection. Therefore, one might assume that there was another point yet unclear along CPT-induced signaling pathways where both chemicals interacted.

A synergistic interaction was observed with the moderate and high concentrations of selenite (5 and 50 μM) and almost the entire concentration range of CPT (Fig. 1D, 1E). This finding is consistent with the results of others who have shown that selenium enhances the chemotherapeutic effect of microtubule inhibiting Taxol and topoisomerase poisoning Adriamycin on several cancer cell lines [29]. We found

that selenite enhanced superoxide anion levels in treated cultures and potentiated mitochondrial damage (Fig. 3D, 6D). Interestingly, in comparison with CPT-treated cultures, the activation of caspase-3 and final chromatin fragmentation which are regarded as established apoptotic markers were reduced over all followed time intervals. This final effect concerning synergism coupled with the changed pattern of cell death may be at least partially explained by a putative double DNA damaging potential of both employed chemicals. Selenite-generated oxidative stress along with suggested direct DNA damaging [33] and direct CPT-DNA interaction most probably caused severe alterations to DNA molecules and directly or indirectly to mitochondria too, causing also severe insult for cells to undergo classical apoptosis.

The results herein presented clearly show that both selenium and CPT have chemotherapeutic effects in cervical carcinoma cells. Still, both chemicals induce different endpoints in treated cells, with CPT triggering apoptosis and selenium producing a type of cell death combining features of both apoptosis and necrosis. Furthermore, inorganic selenite significantly enhanced antineoplastic potential of CPT in cervical carcinoma cells while turning apoptosis into more necrosis-like cell death. Thus these studies point at importance of selenium as a potential modulator and enhancer of CPTs-based anticancer therapy in nonovarian malignancies. Further studies are therefore warranted to verify the plausibility of this approach in clinical applications.

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