Pro-apoptotic Activity of Cyclopentenone in Cancer Cells

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Abstract. Studies on cyclopentenone prostaglandins (CPPGs), clavulones and other cyclopentenones have shown that these compounds have a significant anticancer activity mediated by their cyclopentenone (CP) chemical moiety. In this study the cytotoxicity against cancer cells of the model compound cyclopent-2-en-1-one (2CP) was investigated. Being a highly water soluble small molecule, 2CP could be an ideal candidate to overcome pharmacological issues related to drug delivery and penetration. Its cytotoxic activity was tested on various melanoma and lung cancer cells. Interestingly, 2CP was both cytotoxic and pro-apoptotic, more pronounced on melanoma cells, at concentrations in the sub-micromolar range. On melanoma cells its mechanism of action was mediated by the mitochondria and the activation of caspase 3.

In vitro studies on cyclopentenone prostaglandins (CPPGs), clavulones and other compounds have revealed that the cyclopentenone (CP) moiety is the fundamental chemical entity for their biological activity (1). Their cytotoxic activity can be mimicked by the simple model compound cyclopent-2-en-1-one (2CP), albeit at higher concentrations (2-7).

The α,β-unsaturated carbonyl group of CP can undergo an addition reaction with the sulfhydryl group of cysteine residues located in reduced glutathione (GSH) or other proteins, inactivating them. Alkylation of crucial cysteine residues can result in a loss of function of the targeted proteins (5). The α,β-unsaturated carbonyl group is a ‘soft’ electrophile so that alkylation of weaker nucleophilic sites located in other macromolecules, such as DNA, is much less likely (8). Nuclear factors have been indicated as molecular targets for CP activity (5). Targets in the mitochondria could also be involved in CP mediated anti-proliferative activity (1). Notably, 2CP can elicit an antiviral activity mediated by the activation of heat-shock proteins in hosting cells (2).

Despite all these promising indications, so far and to the best of our knowledge only one study has addressed the effect of 2CP on cancer cells, specifically dealing with the anti-proliferative action of 2CP on one breast cancer cell line (9). In the present work, the cytotoxicity of 2CP on various cancer cell lines was evaluated.

Cancer cells derived from highly malignant diseases, in particular advanced melanoma and non-small cell lung cancer (NSCLC) cell lines, were chosen. Advanced, metastatic melanoma is almost uniformly a fatal disease and very few effective pharmacological options are available for its therapy. Systemic therapy remains unsatisfactory, inducing complete durable responses in a small minority of patients. Novel agents against melanoma are urgently needed (10). For late stage NSCLC current therapies are characterised by low efficacy and high toxicities. Despite the fact that many therapeutic options are under development, the need for lower toxicity profile agents is still a research priority (11).

Materials and Methods

Cell lines. The study was performed on four melanoma cell lines (M14, M66, M79 and Skmel-2) and three NSCLC cell lines (ChaGo-K1, CAEP and RAL). The M14 and Skmel-2 cell lines, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), while M66 and M79 cell lines were isolated in our laboratory from primary and metastatic lesions of two melanoma patients. ChaGo-K1, a bronchiogenic cell line was obtained from the ATCC while CAEP and RAL, derived from a human epidermoid carcinoma and adenocarcinoma, respectively, were obtained and characterized in our laboratory (12). The cell lines were maintained as a monolayer at 37°C and subcultured weekly. The culture medium was composed of DMEM/HAM’S F12 (1:1) supplemented with fetal calf serum (10%), glutamine (2 mM), non-essential aminoacids (1%) (Mascia Brunelli s.p.a., Milan, Italy), and insulin (10 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). The cells were used in the exponential growth phase in all the experiments.

Drugs. The 2CP (Sigma Aldrich) was provided as a liquid highly soluble in water. It was serially diluted with distilled water to concentrations 10 times higher than those tested, stored at –70°C before being freshly diluted in culture medium for each experiment.
In vitro chemosensitivity assay. The sulforhodamine B (SRB) assay was used according to the method of Skehan et al. (13). Briefly, the cells were collected by trypsinization, counted and plated at a density of 5,000 cells/well in 96-well flat-bottomed microtiter plates (100 μl of cell suspension/well). The experiments were run in triplicates, and each trial was repeated three times. Eighteen to 24 h after plating (a sufficient time for exponential growth recovery), 100 μl of culture medium containing the 2CP was added to the wells. After drug exposure, the cells were fixed with 50% trichloroacetic acid at 4°C (50 μl/well, final concentration 10%) for 1 h, washed with tap water, stained with 0.4% SRB in 1% acetic acid (100 μl/well) for 30 min and washed again with 1% acetic acid to remove unbound stain. The plates were air-dried, protein-bound stain was solubilized with 100 μl of 10 mM unbuffered Tris base and the optical density (OD) of each well was determined at a wavelength of 490 or 540 nm using a fluorescence plate reader.

The growth inhibition and cytotoxic effect of the drugs were calculated according to the formula reported by Monks et al. (14):

\[(\text{OD}_{\text{treated}} - \text{OD}_{\text{zero}})/(\text{OD}_{\text{control}} - \text{OD}_{\text{zero}}) \times 100\%\]

If \(\text{OD}_{\text{treated}} \geq \text{OD}_{\text{zero}}\), cell killing had occurred and the following formula was used:

\[(\text{OD}_{\text{zero}} - \text{OD}_{\text{treated}})/(\text{OD}_{\text{zero}}) \times 100\%\]

\(\text{OD}_{\text{zero}}\) depicts the cell number at the precise moment of drug addition, \(\text{OD}_{\text{control}}\) reflects the cell number in untreated wells and \(\text{OD}_{\text{treated}}\) reflects the cell number in treated wells on the day of the assay. The data reported in the graphs are averages of the reading of three independent wells, with the relative standard deviation.

Western blotting. The cells were lysed and cellular proteins were denaturated, separated on a 10% SDS-polyacrylamide gel and then electroblotted onto Hybond-C extra membrane (Amersham Pharmacia Biotech, Cologno Monzese, Italy). The membranes were stained with Ponceau S (Sigma Aldrich) to verify sample loading and then incubated for 2 h at room temperature with PBS containing 0.1% Tween and 5% non-fat dry milk. The membranes were probed overnight at 4°C with a polyclonal antibody against caspase-3 diluted 1:500 (Cell Signalling Technology, Inc., Beverly, MA, USA), after which horseradish peroxidase-conjugated secondary antibody diluted 1:1000 (Dako Corporation, Glostrup Denmark) was added. The bound antibodies were detected by enhanced chemiluminescence (ECL) using an ECL kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Flow cytometric analysis. TUNEL assay. The cells were trypsinized, fixed in PBS + 1% paraformaldehyde on ice for 15 min, suspended in ice cold ethanol (70%) and stored overnight at –20°C. They were then washed twice in PBS and incubated with 50 μl of TUNEL reaction mixture (Roche Diagnostic GmbH, Mannheim, Germany) containing terminal deoxynucleotidyl transferase (TdT) and FITC-conjugated with uridine 5'-triphosphate deoxynucleotide (FITC-conjugated (dUTP)), in a humidified atmosphere for 60 min at 37°C in the dark. The samples were then washed in PBS containing 0.1% Triton X-100, counterstained with 3 μg/ml of propidium iodide (Sigma Aldrich) and 10 K units/ml of RNase (Sigma Aldrich) for 30 min at 4°C in the dark and finally analyzed using a FACS Vantage flow cytometer (Becton Dickinson, San Diego, CA, USA). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10,000 events were recorded.

Mitochondrial membrane potential (ΔΨ) depolarization assay. The cells were harvested, washed once in PBS and then immediately incubated in JC-1 Working solution (BD Biosciences Pharmingen, San Diego, CA, USA) for 10 min in a humidified atmosphere at 37°C in the dark. They were then washed and resuspended in 1X Assay Buffer (BD Biosciences Pharmingen) and analyzed by FACS. Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 15,000 events were recorded.

Fluorescence microscopy with propidium iodide (PI). The cells were trypsinized, washed twice in PBS and then incubated for 2 h with PI at a concentration of 5 μg/ml. The cells were then washed with PBS and observed by means of fluorescence microscopy using an Axioskop 40 microscope (Carl Zeiss, NY, USA). The cells were stained red by PI and apoptotic cells were identified by the characteristic blebs of their nuclei.

Results

Cytotoxic effect of 2CP. The data illustrated in Figures 1a and 2a showed that 2CP had a strong anti-proliferative activity against all the cell lines tested after a standard exposure time of 24 h.

The growth-inhibitory effect after 24 h treatment with 2CP was more pronounced on the melanoma than on the NSCLC cell lines. On the melanoma cells, 2CP showed a strong cytotoxic effect in all the cell lines examined. In particular IC_{50} values were reached between 0.08 and 0.4 μM and the cytotoxic effect was attained at doses higher than 0.4 μM. On the lung cancer cells, the compound showed a modulation of its effect depending on the cell line used, in particular CAEP and RAL were more resistant to the drug effect than ChaGo-K1. A IC_{50} was reached at doses lower than 0.01 μM and the cytotoxic effect was reached at doses higher than 0.2 μM, for the ChaGo-K1 cells.

To evaluate any eventual reversibility of the cytotoxic activity of 2CP, another set of measurements were taken where exposure to 2CP was followed by 24 h of washout in 2CP free culture medium. The data presented in Figures 1b and 2b, when compared to those in the corresponding Figures 1a and 2a, clearly indicated a lack of re-growth effect after the removal of 2CP, thus confirming a permanent effect of 2 CP on the cells. The percent reduction of cell viability after the removal of 2CP, thus confirming a permanent effect of 2 CP on the cells. The percent reduction of cell viability was not affected by a restorable inhibitory effect on cell growth, at least over the time examined in our setting.

To discriminate between an anti-proliferative effect and a true cytotoxic effect, the cells were examined under the fluorescence microscope. Figure 3 shows an exemplificative microscope field. Propidium iodide staining revealed DNA condensation and nuclear fragmentation typical of apoptotic processes, with apoptotic blebs present in many of the cells.
when exposed to 2CP for 24 h. Similar nuclear morphology was observed even after an additional 24 h of washout.

Analysis of apoptosis induction. As shown in Table I, 24 h exposure to 2CP induced a significant rise ($p<0.05$) in apoptosis for all the melanoma cell lines as analysed by flow cytometry. The apoptosis continued to increase with time, regardless of 2CP washout, as shown in Table I.

Skmel-2 and M14 were the most sensitive cell lines reaching a level of 69.1% and 63% of apoptotic cells respectively. In the Skmel-2 cells apoptotic induction was more rapid reaching...
a high level after only 24 h treatment, while in the M14 cells apoptotic induction was more progressive. Conversely the M66 and M79 cell lines were shown to be more resistant to 2CP activity with a level of 39.5% and 29.7% of apoptotic cells after drug treatment respectively.

Interference on apoptotic-related pathway in M14. To investigate, the mechanism of pro-apoptotic activity of 2CP on the melanoma cells at a molecular level, the induction of procaspase-3 cleavage was measured by Western blot after 24 h of 2CP exposure or when this treatment was followed by 24 or 48 h of washout time in the M14 melanoma cell line. As shown in Figure 4, 2CP was able to induce detectable caspase 3 cleavage, indicating that a mitochondrial mediated apoptosis pathway was involved in the 2CP cytocidal effect on the cancer cells.

Mitochondrial collapse evaluation. To confirm an involvement of mitochondria in 2CP induced apoptosis, the ΔΨ collapse was also measured. The FL2-H fluorescence counts are reported in Figure 5, in which the cells with functional mitochondrial membranes are shown as empty histograms whereas the cells with non functional mitochondrial membranes are shown as greyed histograms. A decrease of functional mitochondria was already apparent after 24 h 2CP treatment (Figure 5, 1). A progressively more marked increase in the percentage of cells with a collapse of ΔΨ was then detectable as the time increased (after an additional 24 or 48 h), even if the washout of 2CP had been performed. The effect of the 2CP 24-h treatment on cell mitochondria could therefore be considered chronic in nature.

Discussion

The cytotoxicity data obtained in this study indicated that 2CP was able to inhibit the growth of both melanoma and NSCLC cell lines of various types. All the melanoma cell lines tested were highly sensitive to the activity of 2CP with an inhibitory effect starting at sub-micromolar concentration ranges. It must be emphasized that our data were obtained after 24 h continuous exposure in the culture media and thus did not evaluate the minimum time required to induce the cytotoxic effect of 2CP. Shorter exposures were not properly evaluated in this study, despite preliminary (unpublished) data indicating an inverse dependence of the 2CP cytocidal effect to the initial exposure time.

Considering the high water solubility of 2CP and its small molecular size, two important features that could minimize drug delivery issues, our data indicated that 2CP could be a promising candidate for further anticancer research.

Our in vitro setting simulated a 24 h continuous in vivo infusion. This observation must be considered when translating these data to in vivo strategy settings. In fact, pharmacokinetic variations obtained with bolus administration instead of continuous infusion could result in ineffective in vivo treatments, which has been observed in well-known cases of anticancer treatment with small water soluble molecules, as in a recent report on 3-bromopyruvate (15).

From a mechanistic point of view, our data indicated mitochondria as a target of 2CP pro-apoptotic activity on cancer cells, in particular on the melanoma M14 cells. This is inferred from the observation of nuclear condensation and fragmentation, as shown by fluorescence microscopy using propidium iodide staining (Figure 3), caspase 3 fragmentation (Figure 4) and mitochondrial collapse data (Figure 5).

Mitochondrial mediated induction of apoptosis for cell killing is considered a preferential option for anticancer strategies, since it is more selective than other cell killing strategies (16). In fact, cancer cells are often characterised by molecularly and functionally altered mitochondria and an altered ion channels-mitochondrial axis when compared with corresponding healthy ones (17-19). In addition, our analysis of apoptosis (Table I) indicated that after the mitochondrial involvement, a slow but progressive cytocidal
effect was triggered in the melanoma cells in culture. This slow irreversible effect could be particularly important when formulating an anticancer strategy.

Since specific targets for anticancer activity of 2CP were unknown, cell lines that harbour differential expression of various molecular markers (20,21) were selected. Empirically, the melanoma were more sensitive to the 2CP cytocidal effect than the NSCLC cell lines. From the data available to us, it was not possible to discriminate the reasons for this differential response at the molecular level.

In a translational perspective, the elevated sensitivity of melanoma cancer cells could be exploited for topical treatments of melanoma lesions in vivo. A possible model to verify this hypothesis could be a chemically induced melanoma in mice. However, since advanced metastatic forms of melanoma are life threatening in humans, systemic treatments should also be verified in metastatic melanoma implantable lines, for example the well known BF16 metastatic melanoma model in mice. These in vivo trials are recommended as highly promising, but cannot be currently undertaken in our laboratory.

From a toxicology perspective, differential cytotoxicity in non-cancerous proliferating cells must be evaluated, to provide a rational for the potential side-effects of 2CP. Endothelial umbilical cell cytotoxicity has been evaluated by Vosseler and co-workers (22). In their experimental setting, 2CP reduced cell viability in a dose dependent manner and significantly induced apoptosis in Human Umbilical Vein Endothelial (HUVEC) cells with a concentration as low as
0.25 μM. Potential toxicity could thus be expected in vivo for systemic administrations. The pharmacokinetic and pharmacodynamic profiles of this chemical should therefore be evaluated scrupulously.

Conclusion

The simple model compound 2CP, in sub-micromolar concentrations, is cytotoxic and pro-apoptotic in vitro against cancer cells derived from human malignant tumors, triggering progressive and irreversible mitochondrial mediated apoptotic events. It is a very promising candidate for further anticancer research and in vivo settings are warranted.

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