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# Oxaliplatin (L-OHP) treatment of human myeloma cells induces *in vitro* growth inhibition and apoptotic cell death

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**Abstract**

Oxaliplatin (L-OHP), a diaminocyclohexane platinum derivative, is an active and well tolerated anticancer drug which is presently used in the treatment of gastrointestinal tumours. Since the efficacy of L-OHP in the treatment of multiple myeloma (MM) has not yet been evaluated, we studied the antiproliferative activity of this compound *in vitro* in a panel of MM cell lines (XG1, XG1a, U266 and IM-9). We found that L-OHP inhibited the growth of MM cells at therapeutically achievable concentrations ($IC_{50}$: 5–10 μM after 24 h of exposure) and was more active than Cisplatin (CDDP) or Carboplatin (CBDCA). The activity of L-OHP was apparently not affected by interleukin-6 (IL-6), the major growth and survival factor of MM cells. We also found that L-OHP induced apoptotic cell death. We demonstrated that the combination of L-OHP with Dexamethasone (Dex) resulted in the enhancement of the anti-myeloma effects. L-OHP and Dex both induced poly adenosine diphosphate (ADP)-ribose polymerase (PARP) cleavage and this induction was enhanced by the combined treatment. L-OHP-induced apoptosis correlated with caspase-3 cleavage, but this correlation could not be demonstrated in Dex-treated cells. Taken together, these *in vitro* results provide a rationale for the experimental use of L-OHP in the treatment of MM patients and suggest therapeutic combinations of potential value. 

*Keywords:* Oxaliplatin; L-OHP; Dexamethasone; Multiple myeloma; Myeloma cell lines; Apoptosis; IL-6; Sant7; Caspase-3; PARP

## 1. Introduction

Multiple myeloma (MM) is a clonal B-cell neoplasm that involves terminally differentiated B-cells [1]. During the past three decades, the standard treatment has not substantially changed [2]. Recently, high-dose chemotherapy followed by haematopoietic stem-cell support resulted in an increased response rate and prolonged survival in a selected subgroup of patients [3]. However, the disease unavoidably relapses and myeloma cells become drug-resistant. The disappointing results of the presently available treatments have resulted in the need for an investigation of new drugs for the treatment of MM.

Oxaliplatin (L-OHP) is a third-generation platinum coordination complex of the 1,2-diaminocyclohexane family [4,5]. Among the platinum derivatives, L-OHP appears particularly promising because it lacks the nephrotoxicity associated with Cisplatin (CDDP) and the myelosuppression associated with Carboplatin (CBDCA) treatments. L-OHP is also effective against tumour cells with an intrinsic or acquired resistance to CDDP, specifically due to altered DNA mismatch repair [6–8]. L-OHP is presently used in the treatment of colorectal cancer and has also shown promising efficacy in preclinical studies targeted on different neoplasms, including solid and haematopoietic malignancies [4,5,7,9–11], but its activity against MM cells has not yet been analysed.

On this basis, we have evaluated the antiproliferative and apoptotic effects of L-OHP in a panel of human

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MM cell lines with differing degrees of sensitivity to interleukin-6 (IL-6). This cytokine is the major growth and survival factor for MM [12]. Moreover, we have studied whether dexamethasone (Dex), which is a drug commonly used in the treatment of MM, could produce an enhancement of the anti-tumour activity of L-OHP on MM cells. Finally, we evaluated the involvement of the caspase-3 protease and poly adenosine diphosphate (ADP)-ribose polymerase (PARP), which are executors of apoptosis [13], in the cell death of MM cells induced by L-OHP and Dex treatment.

## 2. Materials and methods

### 2.1. Reagents

Oxaliplatin, a third-generation antineoplastic platinum coordination complex of the 1,2-diaminocyclohexane family (trans-L-dach (1R, 2R-diaminocyclohexane) oxalatoplatinum, L-OHP), was purchased from Sanofi Winthrop (Gentilly Cedex, France). CDDP and CBDCA were purchased from Bristol-Myers Squibb (Latina, Italy). IL-6 was purchased from PeproTech EC Ltd (London, UK). Dex (Soldesam) was purchased from Laboratorio Farmacologico Milanese (Varese, Italy). The IL-6 receptor (IL-6R) super-antagonist Sant7 was kindly provided by Prof. G. Ciliberto (IRBM, Pomezia, Italy) [14]. Sant7 is a molecular variant of IL-6 which binds with high affinity to the IL-6Rα chain and prevents the binding and dimerisation of the gp-130 chain inhibiting the IL-6-elicited signal transduction. Rabbit polyclonal antibodies against PARP and cleaved caspase 3 (17 KDa) were purchased from New England Biolabs (Beverly, MA, USA).

### 2.2. Cell lines and tissue culture

The IL-6-dependent myeloma cell line XG-1 [15] was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, $5\times10^{-5}$ M (50 μM) β-mercaptoethanol, 100 μg/ml streptomycin and 100 U/ml penicillin in the presence of 4 ng/ml of IL-6. XG-1a, a derivative of the XG-1 cell line, which is partially dependent on exogenous IL-6, due to IL-6 autocrine production, was generated in our laboratory [16,17]. XG-1a cells were cultured in the presence of a low concentration of exogenous IL-6 (0.2 ng/ml), at 37 °C in a 7.5–8% $CO_2$ atmosphere. The U266 and the IM-9 human myeloma cell lines, which are independent from exogenous IL-6, were cultured in RPMI 1640 medium supplemented with 15% or 10% fetal calf serum, respectively, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a 5% $CO_2$ atmosphere.

### 2.3. Cell proliferation assays

Cell proliferation studies were performed using MM cells in the presence of increasing concentrations of L-OHP (1–50 μM). Cells were seeded in 24-well plates at a concentration of $5\times10^4$/ml and viability and cell growth were measured by trypan blue exclusion and haemocytometric cell count. A value of 100% was assigned to the untreated control cultures, and the $IC_{50}$ was defined as the concentration of drug that reduced the number of viable cells to 50% of the control cultures. Growth inhibition induced by L-OHP on the MM cells was compared with equimolar concentrations of CDDP and CBDCA. Analysis of the effects of L-OHP on XG-1a cells, the IL-6 autocrine cell line, was also carried out in the presence of the IL-6R super-antagonist Sant7 (200 ng/ml). Growth inhibition was also analysed after combined treatment with L-OHP ($IC_{50}$ dose) and Dex ($10^{-5}$ M).

### 2.4. Flow cytometric analysis of apoptosis

Apoptotic cell death was analysed by Annexin-V fluorescein isothiocyanate (FITC) staining and the Mebstain technique, a modification of the terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labelling (TUNEL) method. The Annexin-V FITC binds to phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis [18]. Labelling of apoptotic cells was performed using an Annexin-V kit (MedSystems Diagnostics, Vienna, Austria). Briefly, cells were incubated with Annexin-V-FITC in binding buffer (provided by the manufacturer) for 10 min at room temperature, washed, and resuspended in the same buffer as described by the manufacturer. The analysis of the apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson).

The Mebstain method (Immunotech, Marseille, France) [19] measures the incorporation of fluorescein-dUTP (FITC-dUTP) catalysed by TdT, thereby enabling the detection of DNA fragmentation into nucleosome multimers during programmed cell death. Briefly, $1–2\times10^6$ cells were washed and fixed with 200 μl of 4% paraformaldehyde (PFA) (v/v) at 4 °C for 30 min. Thereafter, cells were washed and permeabilised in 70% ethanol (v/v) at −20 °C for 30 min. Cells were washed again and resuspended in a solution containing FITC-dUTP and TdT for 1 h at 37 °C. Finally, after a further washing, cells were analysed by flow cytometry.

### 2.5. Western blot analysis of caspase-3 and PARP

Whole-cell pellets were solubilised with a lysis buffer containing 50 mM Tris–HCl pH 6.8, 140 mM NaCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM

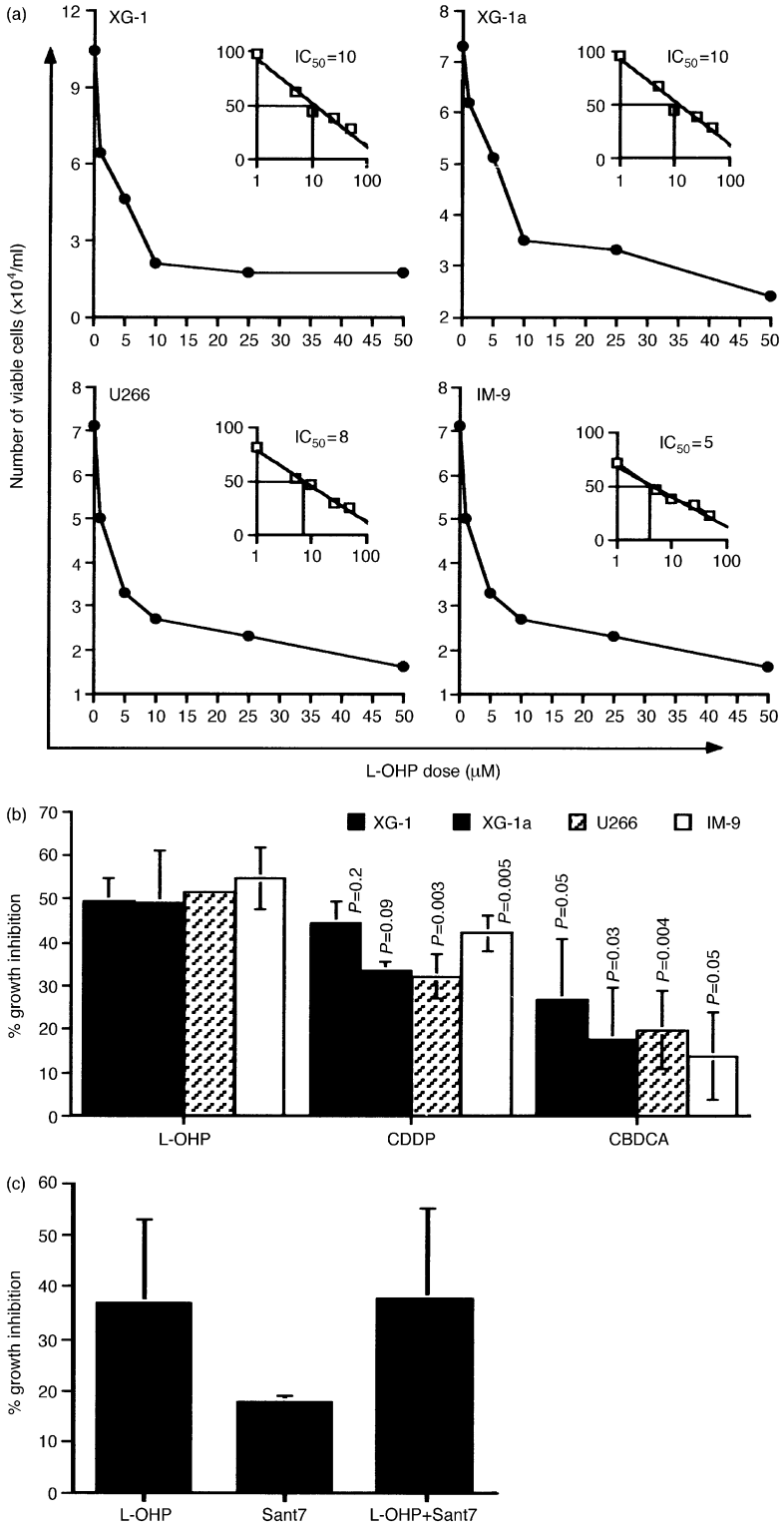


Fig. 1. Effects of Oxaliplatin (L-OHP) on multiple myeloma (MM) cell proliferation; relevance of the Interleukin-6 (IL-6) pathway. In all cell proliferation assays, $5\times10^4$ cells/ml were seeded into 24-well plates and exposed to different drug concentrations. The number of viable cells was determined by haemocytometric cell count after 24 h of culture and by trypan blue exclusion. Data are expressed as Means±Standard Deviations (S.D.) of at least three separate experiments. In (a) the dose-related activity of L-OHP on the MM cells is shown. The logarithmic curve and the $IC_{50}$ of cell growth are also shown. In (b), a comparative analysis of growth inhibition following L-OHP, Cisplatin (CDDP) and Carboplatin (CBDCA) treatment is shown. Cell lines have been exposed for 24 h to equimolar concentrations of the drug ($IC_{50}$ for L-OHP). The statistical significance of differences between L-OHP, CDDP or CBDCA treatments for each MM cell line is reported. In (c), growth inhibition has been evaluated in IL-6 autocrine XG-1a cells, cultured with low doses of exogenous IL-6 (0.2 ng/ml), in the presence of L-OHP (10 μM for 24 h) and/or the IL6 receptor antagonist Sant7 (200 ng/ml). Data are expressed as Means±S.D. of at least three separate experiments.

ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′- tetracetic acid (EGTA), 1 mM sodium vanadate, 0.2 mM phenylmethylsulphonylfluoride (PMSF) and 0.5% Nonidet (v/v) P-40. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Milan, Italy). Equal amounts of cell lysate (50 μg) were separated by 10% sodium dodecyl sulphate–polyacrylamide (v/v) gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Amersham, Aylesbury, UK). After blocking with 5% non-fat dry milk (w/v) containing 0.05% Tween (v/v), membranes were incubated with the appropriately diluted anti-PARP or anti-cleaved caspase-3 primary antibody. Then, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody. Proteins were detected by the enhanced chemiluminescence detection system (Amersham, Aylesbury, UK).

### 2.6. Caspase-3 activity colorimetric assay

Caspase-3 activity was also determined using the Caspase-3 Colorimetric Assay Kit (Alexis Biochemicals, Italy) according to the manufacturer's protocol. The assay is based on a spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate Asp-Glu-Val-Asp-pNA (DEVD-pNA). This kit provides a simple means for assaying the activity of caspases that recognise the sequence DEVD. Briefly: cells ($1–5\times10^6$) were homogenised in 50 μl of lysis buffer, incubated for 10 min on ice and centrifuged at 10 000*g* for 1 min at 4 °C. The protein content of the supernatant was quantified using the Bio-Rad protein assay kit. The caspase activity was assayed directly in a 96-well plate using 50–200 μg of proteins. 50 μl of 2× reaction buffer containing 10 mM dithiothreitol (DTT) and 5 μl of 4 mM DEVD-pNA substrate (200 mM final concentration) were added to each of the samples. The samples were incubated for 2 h at 37 °C, the absorbance of each well was measured at 405 nm Wavelength (WL) by a microtitre plate reader. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity. Background reading from cell lysates and buffers was subtracted from the readings of both induced and uninduced samples before calculating the fold increase in caspase-3 activity.

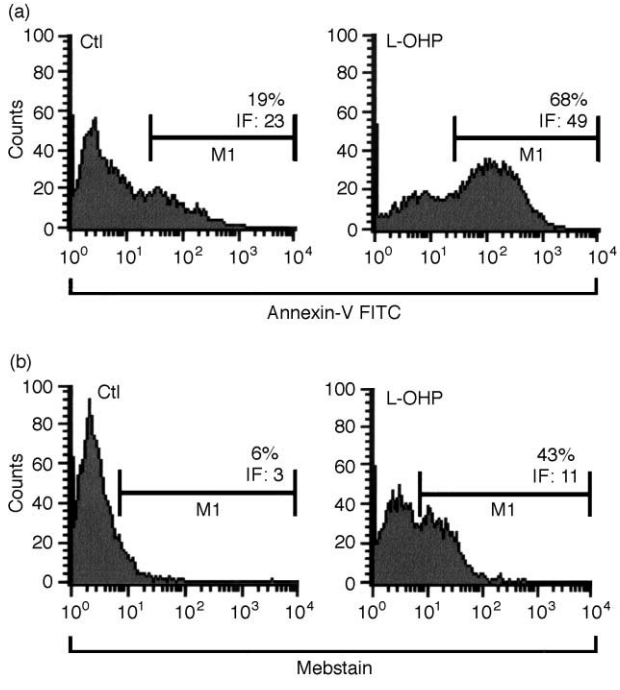


Fig. 2. Apoptotic effect of Oxaliplatin (L-OHP) treatment of IM-9 multiple myeloma (MM) cells. Apoptosis induced by L-OHP (10 μM for 24 h) was performed in parallel with the Annexin-V staining (a) and the Mebstain method (b) in IM-9 cell line. Percentage of stained cells (%) and mean channel intensity of fluorescence (IF) are indicated in each graph. Ctl, control; FITC, fluorescein isothiocyanate.

### 2.7. Statistical analysis

Results are expressed as Mean ± Standard Deviation (S.D.). The statistical significance of differences between the experimental points was analysed using the *t*-test; differences were considered significant when $P \leqslant 0.05$.

## 3. Results

### 3.1. Growth inhibition, relevance of the IL-6 growth survival pathway and induction of apoptosis by L-OHP in the MM cells

The antiproliferative activity of L-OHP has been evaluated in four human myeloma cell lines. Fig. 1a shows the dose-related effect of L-OHP in the 1–50 μM range after 24 h of exposure. The L-OHP $IC_{50}$ for all cell lines was found range from 5 to 10 μM. The activity of L-OHP was compared with the effect of CDDP or CBDCA on each cell line. Fig. 1b shows that L-OHP, at equimolar concentrations, is more effective than CDDP in two out of the four MM cell lines and more effective than CBDCA in inducing growth inhibition in all of the MM cell lines.

We have investigated whether the IL-6-mediated growth/survival pathway could antagonise the antiproliferative effect of L-OHP. The comparative analysis of the MM cell lines with differing sensitivities to IL-6, including the exogenous IL-6-dependent (XG-1), the exogenous IL-6-partially dependent and with IL-6 autocrine production (XG-1a), and the IL-6-independent (U266 and IM-9) cells did not show significant differences in the antiproliferative effects of this compound (data not shown). Moreover, the addition of the IL-6 receptor super-antagonist Sant7 [14] (200 ng/ml), which prevents the assembly of functional IL-6 receptor complexes and inhibits the IL-6-mediated receptor and

post-receptor events, did not produce any significant enhancement of growth inhibition ($P=0.9$) of the XG-1a cells in the presence of 10 μM L-OHP and low doses of exogenous IL-6 (0.2 ng/ml) (Fig. 1c). These findings suggest that the L-OHP-cytotoxic activity is not antagonised by the IL-6-mediated growth/survival pathways in MM cells.

To clarify the mechanisms of L-OHP-induced growth inhibition, we analysed the amount of apoptosis occurring in the MM cells after treatment. Fig. 2 shows a representative experiment in which the amount of apoptosis was evaluated in IM-9 cells by measuring both Annexin-V FITC staining (a) and the FITC-dUTP incorporation (b). The flow cytometric analysis by the two techniques revealed similar results indicating that apoptosis plays a substantial role in the antimyeloma activity of L-OHP. Apoptosis was also detected in the U266, XG1 and XG1a MM cell lines (data not shown).

### 3.2. Effect of the L-OHP and Dex combination on MM cells. Activation of caspase-3 and cleavage of PARP

We evaluated the interaction of L-OHP with Dex, which is an active and commonly used agent in the treatment of MM. The combination of L-OHP and Dex resulted in a more than additive antiproliferative effect in all of the MM cells (Fig. 3a). The antiproliferative effect of L-OHP/Dex combination was paralleled by an increased apoptotic activity as shown in a representative experiment on IM-9 cells (Fig. 3b). We found that treatment with both compounds increased the expression and cleavage of PARP, a key enzyme of the apoptotic process [13], as demonstrated by western blot analysis which showed increased expression of the 116 kD proenzyme and the appearance of the 89 kD cleavage product in treated cells (Fig. 3c). The combined treatment with L-OHP and Dex resulted in

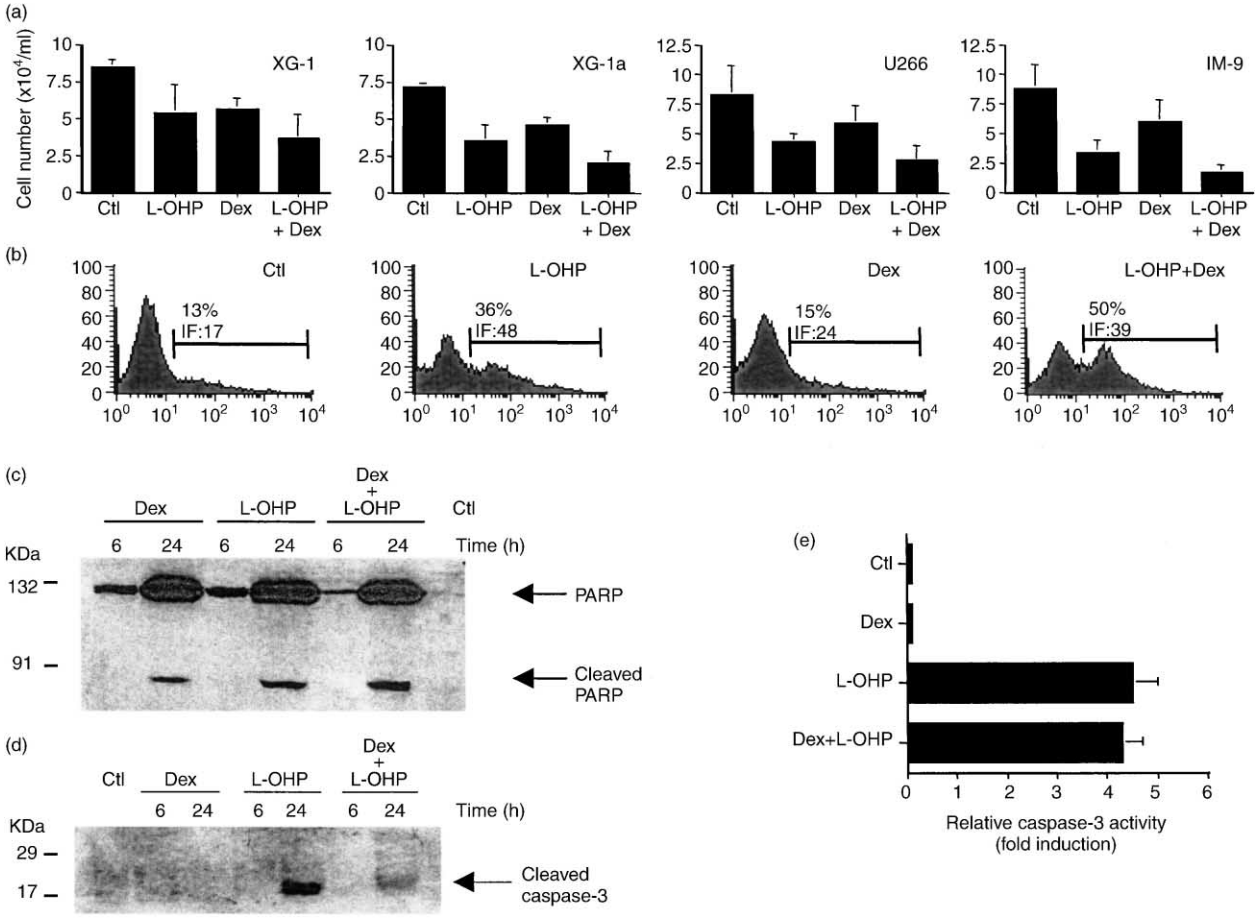


Fig. 3. Effect of the Oxaliplatin (L-OHP)/Dexamethasone (Dex) combination on IM-9 cell growth and apoptosis; involvement of poly adenosine diphosphate (ADP)-ribose polymerase (PARP) and caspase-3. In panel (a) the antiproliferative activity of L-OHP and/or Dex on MM cells is shown. The number of viable cells was determined by haemocytometric cell counting after 24 h of culturing and by trypan blue exclusion. Data are expressed as Means±Standard Deviations (S.D.) of at least three separate experiments. The apoptotic effect of L-OHP and/or Dex on IM-9 MM cells was assayed by Annexin-V staining (panel b). The percentages of apoptotic cells (%) and the mean fluorescence intensity (IF) are indicated above the marker windows. In panel (c), the effect of L-OHP and/or Dex on PARP activation is demonstrated by western blotting analysis after 6 and 24 h of treatment. The effect of L-OHP and/or Dex on caspase-3 activation, at the same time points, is showed by western blotting analysis in panel (d) and by DEVDase colorimetric assay in panel e. Ctl, control.

enhanced PARP cleavage. The maximal effect could be detected after 24 h of L-OHP/Dex exposure when more than 50% of the cells underwent apoptosis. As compared to 6 h and 12 h time points (data not shown). We also found that caspase-3 was cleaved/activated after 24 h of exposure to L-OHP, but was not affected by Dex, as demonstrated by western blot and DEVDase colorimetric assays (Fig. 3d and e). Moreover, the kinetics of caspase-3 activation by L-OHP clearly correlated with the cleavage of PARP, which is considered a caspase-3 substrate [20]. PARP cleavage on myeloma cells therefore appears to be induced by different activation pathways, caspase-3-dependent following L-OHP treatment and caspase-3-independent following Dex treatment.

These results demonstrate that L-OHP combined with Dex strongly inhibits myeloma cells and suggest that these compounds may activate different death pathways which finally converge in PARP activation and DNA cleavage.

## 4. Discussion

In this paper, we report that the *in vitro* exposure of human MM cells to clinically achievable concentrations of L-OHP exerts a strong antiproliferative effect and induces apoptotic cell death. This is the first report of an anti-myeloma activity induced by L-OHP. Our results highlight the potential clinical value of this third-generation CDDP analogue in the treatment of MM.

Platinum-derivative compounds are thought to exert their antiproliferative and apoptotic effects through the formation of various types of DNA lesions [21,22]. During biotransformation, L-OHP retains the D1,2-diaminocycloexane ligand in the reactive entities, resulting in DNA adducts more effective at inhibiting DNA synthesis compared with other platinum-derivatives (CDDP/CBDCA) which yield common *cis*-diammine-platinum adducts [21]. The adducts formed by L-OHP exert a greater cytotoxicity than those induced by CDDP or CBDCA [4,5,7,9–11]. Interestingly, preclinical and clinical studies have suggested that L-OHP may offer therapeutical advantages for a variety of malignancies with either intrinsic or acquired CDDP resistance [4,5,7,9–11]. Notably, L-OHP exerts a well defined antitumour effect against colorectal cancers which are almost completely unresponsive to CDDP or CBDCA [9]. The evidence that L-OHP may exert a specific antitumour effect, the overcoming of CDDP resistance and the lack of nephrotoxicity and myelosuppression, which are commonly associated with CDDP and CBDCA treatments, strongly suggest that L-OHP may be a promising new compound for the therapy of MM patients. In fact, due to their dose-limiting nephro- and myelo-toxic side-effects and due to the frequent association of MM with renal failure and myelosuppression, the most common chemotherapeutic regimens do not usually include platinum complexes such as CDDP or CBDCA. In this light, L-OHP may offer an alternative approach for the treatment of MM. Peripheral neurosensory toxicity is of some concern but, considering the widespread use of L-OHP in gastrointestinal malignancies, it is rarely dose-limiting [23].

One specificity of MM cell growth is the bone marrow micro-environment (BMM) [12], where IL-6 appears to exert a major role as a paracrine/autocrine growth promoting and survival factor for MM cells. In this context, we have studied the effects of L-OHP on a panel of MM cells with differing sensitivities to IL-6. Significant differences among the MM cell lines were not detected. XG-1a cells [16,17] were also used, which secrete detectable amounts of IL-6 and require only low concentrations of exogenous IL-6. It was shown that the disruption of the IL-6 pathway by the IL-6 receptor super-antagonist Sant7 does not modify the L-OHP-induced growth inhibition of the XG1a MM cells, Taken together, these findings suggest that a functional autocrine/paracrine IL-6 growth/survival pathway does not protect MM cells from L-OHP-induced growth inhibition.

In addition, we have studied the antimyeloma effect induced by L-OHP in combination with Dex. Glucocorticoids, which are active in the treatment of MM, are not nephrotoxic or myelosuppressive and are particularly useful in advanced MM patients who have a poor renal and bone marrow reserve [2]. Therefore, Dex represents an excellent drug for combined treatment regimens. It was observed that the combination of L-OHP and Dex produced increased antimyeloma effects. Moreover, we demonstrated that L-OHP and/or Dex induced apoptotic cell death in the MM cells. To examine the mechanisms by which apoptosis is induced by the two compounds, the expression and cleavage of PARP and caspase-3, key enzymes in the apoptosis process, were evaluated. Notably, it was found that the induction of apoptosis by both compounds in IM-9 cells correlated with enhanced PARP expression and cleavage/activation. However, the upstream death pathways that induced PARP were divergent, since caspase-3 was activated by L-OHP treatment but was not affected by Dex treatment.

In conclusion, our findings indicate that L-OHP is a promising agent for the treatment of human MM. The positive interaction of L-OHP with Dex suggests a novel therapeutic combination that deserves clinical evaluation. The involvement of different death pathways induced by L-OHP and Dex treatments warrants further investigation for the development and molecular design of individually tailored therapeutic approaches.

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