Curcumin Stimulates Reactive Oxygen Species Production and
Potentiates Apoptosis Induction by the Antitumour Drugs Arsenic
Trioxide and Lonidamine in Human Myeloid Leukaemia Cell Lines

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Running Title Page

Running title: Curcumin increases arsenic trioxide and lonidamine toxicity

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ABBREVIATIONS: ANT, adenine nucleotide translocator; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; ATO, arsenic trioxide; BSO, L-buthionine-(S,R)-sulfoximine; Cisplatin, CDDP, cis-platinum(II)-diammine dichloride; CsA, cyclosporin A; CML, chronic myeloid leukaemia; DAPI, 4,6-diamino-2-phenylindole; DHE, dihydroethidium; DiIC 1(5), 1,1',3,3',3'-hexamethyleneindodicarbocyanine iodide; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; JNK, c-Jun NH₂-terminal kinase; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK/ERK, mitogen-induced extracellular kinase/extracellular signal-regulated kinase; MnTBAP, Mn(III)tetrakis(4-Benzoic acid)porphyrin chloride; mPTP, mitochondrial transition pore; MTT, 3(4,5-Dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide; NAC, N-acetyl-L-cysteine; pAb, polyclonal antibody; PBS, phosphate buffered saline; PD98059, 2'-Amino-3'-methoxyflavone; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide; ROS, reactive oxygen species; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; z-VAD-fmk, Z-Val-Ala-Asp(OMe)-CH₂F.

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Arsenic trioxide (ATO, Trisenox®) is an important anti-leukaemic drug, but its efficacy is frequently low when used as a single agent. We here demonstrate that the apoptotic action of ATO is greatly increased when combined with subcytotoxic curcumin concentrations in U937 and HL60 human acute myeloid leukaemia cells, and with lower efficacy in K562 chronic myelogenous leukaemia cells. Curcumin exerts similar cooperative effect with the mitochondria-targeting drug lonidamine, while the response is negligible in combination with the DNA-targeting drug cisplatin. Curcumin plus ATO or lonidamine stimulates typical events of the mitochondrial executioner pathway (Bax and Bid activation, cytochrome c release, XIAP down-regulation, caspase-9/-3 activation), and causes mitochondrial transmembrane potential dissipation, which nevertheless represents a late event in the apoptotic response. Curcumin increases anion superoxide production, and its pro-apoptotic action in combination with ATO and lonidamine is mimicked by pro-oxidant agents (2-methoxyestradiol, H₂O₂) and prevented by anti-oxidant agents (MnTBAP, N-acetyl-L-cysteine). Within the assayed time-period (16-24 h), curcumin does not significantly modify p38-MAPK and JNK phosphorylation/activation or NF-κB activity, but greatly stimulates ERK phosphorylation, and decreases Akt phosphorylation. Experiments using MEK/ERK inhibitors (PD98059, U0126) and PI3K inhibitor (LY294002) indicate that ERK activation does not mediate and even restrains apoptosis potentiation, while Akt down-regulation facilitates apoptosis generation. In summary, co-treatment with curcumin may represent a useful manner of increasing the efficacy of ATO and lonidamine as anti-tumour drugs in myeloid leukaemia cells.
Introduction

Arsenic trioxide (ATO, Trisenox®) is an efficacious, clinically established agent for the treatment of relapsed and refractory acute promyelocytic leukaemia (APL) (Wang and Chen, 2008). At low, physiologically tolerable concentrations (0.25-2 μM in plasma), ATO causes APL cytoreduction by inducing terminal differentiation or apoptosis (Miller et al., 2002). Albeit with lower efficacy, ATO also causes apoptosis in other types of leukaemia and solid tumour-derived cells, which opens the possibility of extending the therapeutic application of the drug (Amadori et al., 2005). Nonetheless, the relatively low sensitivity of most tumour cells to ATO would require the generation of sensitizing strategies, to increase drug efficacy and reduce dosage to clinically achievable concentrations. Importantly, ATO is an oxidant-sensitive agent, in such a manner that drug toxicity is exacerbated under conditions of moderate oxidative stress – namely, decreased intracellular reduced glutathione (GSH) content (Dai et al., 1999) and/or increased reactive oxygen species (ROS) accumulation (Yi et al., 2002). Another aspect of interest is the high intrinsic oxidative stress of most cancer cells (Lau et al., 2008), which makes them more vulnerable to pro-oxidant treatments than their normal counterparts. For this reason, agents that generate a moderate oxidant environment may offer potential therapeutic opportunities.

Curcumin, a hydrophobic polyphenol derived from the rhizome of turmeric (Curcuma longa L.), is a safe, well-tolerated and an efficacious chemo-preventive agent, mainly because of its anti-oxidant and anti-inflammatory properties (Anand et al., 2007). Nonetheless, curcumin may also induce apoptosis in tumour cells and/or potentiate apoptosis induction by classical chemotherapeutic drugs, supporting its potential use in anti-cancer therapies (Limtrakul, 2007; Reuter et al., 2008). In fact, in spite of its poor stability and bioavailability (Anand et al., 2007; Basile et al., 2009), the antitumour efficacy of curcumin has been proved in animal models and phase I clinical trials with multiple types of cancer (Anand et al., 2008). As with other plant-derived
polyphenols, the pro-apoptotic action of curcumin has been largely attributed to the inhibition of defensive signalling pathways such as PI3K/Akt and NF-κB (Lin, 2007; Reuter et al., 2008), but other mechanisms may be equally important. For instance, in spite of its normal antioxidant capacity, curcumin may per se stimulate ROS production (Woo et al., 2003; Chan et al., 2006), or potentiate ROS generation by other agents (Javvadi et al., 2008; Kikuchi et al., 2010) in different tumour cell models. Moreover, curcumin may oxidize thiol residues at the mitochondrial transition pore (mPTP), causing mitochondria dysfunction and cell death (Ligeret et al., 2004).

Earlier studies indicated that curcumin attenuates oxidative stress generation and other toxic effects of arsenic in non-tumour cells in culture (e.g., peripheral blood lymphocytes and Chinese hamster fibroblasts) and animal models (Mukherjee et al., 2007; Roy et al., 2008; Yadav et al., 2009, among others). Nonetheless, we recently reported that another phenolic agent, the soy isoflavone genistein, potentiates ATO-provoked apoptosis in leukaemia cell models but not in normal peripheral blood lymphocytes, and that this effect is mediated by genistein-provoked ROS overproduction and activation of ROS-inducible protein kinases (Sánchez et al., 2008). On this ground, in the present work we analyze the capacity of curcumin to modulate ATO-provoked apoptosis in human U937 and HL60 acute myeloid leukaemia (AML) and K562 chronic myelogenous leukaemia (CML) cells. These cell lines are considered as relatively resistant to ATO in comparison to the highly sensitive APL cells (Jing et al., 1999), and hence are idoneous to study possible cooperative effects. For comparison the action of curcumin was also assayed in combination with two other antitumour drugs, namely cisplatin and lonidamine. Cisplatin is a DNA-targeting drug, while lonidamine as well as ATO are classified as mitochondria-targeting drugs, capable of binding the adenine nucleotide translocator (ANT) at the mPTP, causing pore opening and mitochondria dysfunction (Solary et al., 2003; Fantin and Leder, 2006). The obtained results indicate that curcumin greatly cooperates with ATO and lonidamine to induce apoptosis, while the response is negligible in the case of cisplatin. The pro-
apoptotic action of curcumin is mediated by ROS over-production, and at least in the case of ATO also by Akt down-regulation.
Methods

Reagents and Antibodies. All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA). Monochlorobimane and MitoProbe™ DilC1(5) (1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide) Assay Kit for Flow Cytometry were obtained from Molecular Probes (Eugene, OR). Dihydroethidium (DHE, presented as a 5 mM solution in dimethyl sulfoxide) was obtained from Invitrogen, Inc. 4,6-diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany). Recombinant human tumour necrosis factor (TNF)α was obtained from PeproTech EC (London, UK). The kinase inhibitors 2'-Amino-3'-methoxyflavone (PD98059), 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and 2-(4-Morpholiny1)-8-phenyl-4H-1-benzopyran-4-one (LY294002), the caspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk), and Mn(III)tetrakis(4-Benzoic acid)porphyrin chloride (MnTBAP), were obtained from Calbiochem (Darmstadt, Germany). Rabbit anti-human p38-MAPK, phosho-p38-MAPK (Thr180/Tyr182), SAPK/JNK, phosho-SAPK/JNK(Thr183/Tyr185), p44/42 MAPK, phosho-p44/p42 MAPK (Thr202/Tyr204), Akt, phosho-Akt (Ser473), and cleaved caspase-3 (Asp175) polyclonal antibodies (pAbs), were obtained from Cell Signaling Technology Inc (Danvers, MA). Mouse anti-pigeon cytochrome c monoclonal antibody (mAb) clone 7H8.2C12 was obtained from BD PharMingen (San Diego, CA). Mouse anti-human Bcl-2 (100) mAb, rabbit anti-human Bax (N-20), anti-human caspase-9 p35 (H-170), anti-human NF-κB p65 (sc-109) and anti-human Sam 68 (C-20) pAbs, and goat anti-human Bid (C-20) pAb, were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-XIAP (clone 2F1) mAb was obtained from MBL International Corporation (Woburn, MA). Peroxidase-conjugated immunoglobulin G antibodies were obtained from DAKO Diagnostics, S.A. (Barcelona, Spain). All other reagents and antibodies were from Sigma (Madrid, Spain).
Cells and Treatments. U937 and HL60 human AML cells and K562 human CML cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated bovine serum and antibiotics, in a humidified 5% CO2 atmosphere at 37ºC. For experiments, 16-24 h before the initiation of treatments the cell concentration was adjusted at approximately 10^5 cells/ml. Nonetheless, to prevent the cultures from reaching plateau densities or nutrient exhaustion in long-term experiments (72 h), at the second day of treatment they were supplemented with an equal volume of fresh culture medium containing the corresponding drugs.

Stock solutions of curcumin, cyclosporine A (CsA), 2-methoxyestradiol (2-ME), PD98059, U0126, LY294002 (20 mM each), genistein (50 mM), z-VAD-fmk (25 mM), monochlorobimane (200 mM), lonidamine (100 mM), and N-acetyl-L-cysteine (NAC, 3 M), were prepared in dimethyl sulfoxide. A stock solution of cis-platinum(II)-diammine dichloride (cisplatin, CDDP, 3.3 mM) was prepared in distilled water. MnTBAP was dissolved at 100 mM in aqueous base. 3(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was dissolved at 5 mg/ml in phosphate buffered saline (PBS). All these solutions were stored at -20ºC. Stock solutions of DAPI (10 μg/ml) and propidium iodide (PI, 1 mg/ml) were prepared in PBS. DL-buthionine-(S,R)-sufoximine (BSO) was dissolved at 50 mM in distilled water. ATO was initially dissolved in a small amount of 1 N NaOH, and then diluted with PBS to give a final concentration of 10 mM. These solutions were stored at -4ºC.

Flow Cytometry. The analysis of samples was carried out using an FC-500 cytometer analyzer (Beckman Coulter, Hialeah, FL) equipped with two air-cooled argon lasers tuned to 488 nm (for DHE, PI and Annexin V-FITC) and 630 nm (for DilC1(5)). The specific fluorescence signal corresponding to Annexin V-FITC was collected with a 525-nm band pass filter, the signal corresponding to DilC1(5) with a 675-nm band pass filter, and the signals corresponding to DHE and PI with a 620 nm band pass filter. A total of 10^4 cells were scored in each assay.
Measurement of Cell Viability and Apoptosis. The relative number of viable cells in the culture was determined using the MTT colorimetric assay. With this aim, the cells were centrifuged, re-suspended in 1 ml RPMI medium without red phenol containing 0.5 mg/ml MTT, distributed in quadruplicate in 96-well plates, and incubated for 2-3 h at 37°C, after which SDS was added to a final concentration of 2% to dissolve the formazan crystals. Dye absorbance was measured by spectrometry at 595 nm using an iMark™ Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA). Under the used conditions, a decrease in absorbance may reflect inhibition of cell proliferation, increase in cell death, or the sum of both factors. Alternatively or complementary, cells were incubated with trypan blue, and the number of viable cells (excluding the dye) was determined by microscopy examination.

Distinctive characteristics of apoptotic cells were chromatin condensation/fragmentation, reduction in DNA content (sub-G₁), and phosphatidylserine translocation from the inner to the outer layer of the plasma membrane. Chromatin condensation was determined by cell permeabilization followed by DAPI staining and microscopy examination, and reduction in DNA content was determined by cell permeabilization followed by PI staining and flow cytometry examination. This latter method was also used to analyze cell cycle phase distribution. A detailed description of these procedures was presented in preceding publications (Troyano et al., 2003) and hence is omitted here. Phosphatidylserine translocation was estimated by measuring cell surface binding of annexin-V-FITC by means of flow cytometer, using an ApoAlert® Annexin V-FITC kit, (Contech, Mountain View, CA), following the indications of the manufacturer. Cells treated with curcumin but unlabelled with annexin V were included as controls to measure basal drug auto-fluorescence. As a routine, free penetration of trypan blue into non-permeabilized was examined as an indication of loss of plasma membrane integrity (primary or secondary necrosis).
Determination of Mitochondrial Transmembrane Potential (ΔΨm). At the end of treatments the cells were washed twice with PBS and incubated for 30 min at 37°C with RPMI medium containing 1 nM DilC1(5), after which they were washed twice cold RPMI medium containing 1% fetal bovine serum, re-suspended in cold RPMI medium, and the fluorescence analyzed by flow cytometry. Cells incubated with the depolarizing agent carbonyl cyanide 3-chlorophenylhydrazone (50 μM) were used as an internal control.

Determination of ROS Production. At the end of treatments the cells were washed twice with PBS and incubated for 30 min at 37°C with RPMI medium containing 5 μM DHE, a fluorescent probe which specifically detects anion superoxide production. The cells were then washed twice with cold PBS and re-suspended in cold RPMI medium, and the fluorescence analyzed by flow cytometry. Under these conditions, residual curcumin-derived auto-fluorescence was negligible, as determined using DHE-unlabelled cells. Other commonly used fluorescent probes such as dichlorodihydrofluorescein diacetate (H2DCFDA) were avoided, since residual curcumin auto-fluorescence at the required wavelength (525-nm band pass filter) was still evident.

Measurement of GSH Level. The total intracellular GSH content was determined by fluorometry after cell loading with monochlorobimane, following a previously described procedure (Troyano et al., 2001).

Cell Fractionation and Immunoblot Assays. To obtain total cellular protein extracts, cells were collected by centrifugation, washed with PBS, and lysed for 20 min at 4°C in a buffer consisting of 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, 10% (v/v) glycerol and 1% (v/v) Nonidet P-40, supplemented with a protease inhibitor cocktail, 1 mM sodium orthovanadate and 10 mM NaF. After centrifugation at 10 000 x g for 15 min at 4°C, the supernatants were collected. To obtain mitochondrial extracts, aimed at determining Bax translocation to mitochondria, cells were homogenized by
repeatedly passing them throughout a 25-gauge needle. The homogenate was first centrifuged at 1 000 x g for 10 min, and the supernatant centrifuged again at 10 000 x g for 20 min to obtain the mitochondrial fraction, following the previously described procedure (Troyano et al., 2003). Cytosolic extracts, aimed at determining cytochrome c release from mitochondria, and nuclear extracts, aimed at determining p65 NF-κB translocation, were prepared as previously described (Sánchez et al., 2008, and references therein). Fractions of total, mitochondrial, cytosolic or nuclear extracts, containing equal protein amounts, were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected, as previously described (Galán et al., 2000).

**Electrophoretic Mobility Gel Shift Assays.** All experimental conditions, including nuclear extract preparations, oligoprobe preparation and radioactive labelling, binding reaction and electrophoretic separation, were as previously described (Sánchez et al., 2008, and references therein).

**Statistical Analysis.** When convenient, the significance of differences between experimental conditions was examined using the Student’s t test, and when positive indicated by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).
Results

Apoptosis Generation. Fig. 1 shows the capacity ATO (from 2 to 6 μM) and curcumin (from 5 to 20 μM), used alone and in combination, to decrease viability, induce apoptosis and affect growth cycle progression in U937 human AML cells. As indicated in Fig. 1A, 2 μM ATO as well as 5 and 7.5 μM curcumin caused minimal (less than 10%) apoptosis, while moderate apoptosis (20-40%) was obtained with 4-6 μM ATO and 10-20 μM curcumin, as measured at 24 h of treatment by chromatin condensation/fragmentation. On this ground, the concentrations of 2 μM ATO and 7.5 μM curcumin were selected for combined treatments. When used in combination, curcumin and ATO cooperated in more than additive manner to induce apoptosis, as determined by chromatin fragmentation (Fig. 1A), frequency of cells with sub-G1 DNA content (Fig. 1D), and annexin V binding at the cell surface (Fig. 1E); and also cooperated to reduce cell viability, as determined by the MTT assay (Fig. 1B). Time-course assays indicated that significant apoptosis started to be detected at 16 h of treatment in the combined treatments (Fig. 1C). The treatments caused minimal alterations in cell cycle distribution, except for a slight decrease in the G2/M compartment in curcumin-treated cultures (Fig. 1D). Finally, co-treatment with the pan-caspase inhibitor z-VAD-fmk greatly reduced the frequency of cells with fragmented chromatin in curcumin plus ATO-treated cultures (see Fig. 5C), which corroborates that the observed cell death is bona fide caspase-dependent apoptosis.

For comparative purposes, we examined the action of curcumin and ATO, alone and in combination, in HL60 AML and K562 CML cell lines. The effects of the treatments on viability, cycle phase distribution and apoptosis generation in HL60 cells were similar as in U937 cells (Fig. 2A, and results not shown). On the other hand, a higher ATO concentration (10 μM) was required to obtain significant apoptosis as well as cooperation between ATO and curcumin in K562 cells at 24 h of treatment (Fig.
The relative resistance of this cell line against apoptosis was not compensated by a necrotic response, which at 24 h remained below 6%, similar to untreated cells, as revealed by trypan blue assays (results not shown). Nonetheless lower ATO concentrations (5 μM) affected cell proliferation, as manifested by cell accumulation at G2/M (Fig. 2B), with consequent decrease in the number of viable cells, as indicated by the MTT assay (Fig. 2C). Of note, ATO-provoked cell cycle disturbance was a characteristic of K562 cells, since treatment of U937 and HL60 cells with 4 and 6 μM ATO, which as indicated above markedly induced apoptosis (Fig. 1A), did not significantly affect cycle phase distribution within the 24 h time period (results not shown).

Finally, we analyzed the capacity of curcumin to modulate apoptosis induction by lonidamine (25-100 μM) and cisplatin (1-4 μM) in U937 cells. As a rule, 25 and 50 μM lonidamine and 1-2 μM cisplatin caused minimal (less than 10%) apoptosis, while moderate apoptosis (approx. 20-25%) was obtained with 100 μM lonidamine and 4 μM cisplatin, at 24 h of treatment (Fig. 3A). Nonetheless the toxicity of different lonidamine stocks varied considerably, and hence different concentrations had to be adopted for mechanistic assays. When used in combination, curcumin and lonidamine greatly cooperated to induce apoptosis, as manifested by chromatin fragmentation (Fig. 3A), sub-G1 DNA content (Fig. 3C) and cell surface annexin V binding (Fig. 3D), and to reduce cell viability, as manifested by the MTT assay (Fig. 3B). On the other hand, the combination of curcumin plus cisplatin produced lower (less than additive) effects on apoptosis. Of note, the low apoptotic response was not compensated by the generations of necrosis, which was negligible (less than 10%), as revealed by the absence of trypan blue uptake (not shown) and PI uptake in the annexin V/PI assays (Fig. 3D). Nonetheless, in accordance to its property as a DNA-damaging agent, cisplatin affected cell cycle transition throughout the the S and G2/M phases (Fig. 3C), which may explain the reduction in the total number of viable cells, as revealed by the
MTT assay (Fig. 3B). This cell cycle disturbance was attenuated by co-treatment with curcumin (Fig. 3C). In another group of experiments, U937 cells were subjected to long-term treatments (72 h) with 2 μM cisplatin. Under these conditions the drug caused a great decrease in cell number, cell accumulation at G2/M, cell hypertrophy, apoptosis, and necrosis (probably “secondary”, apoptosis-derived necrosis). Co-treatment with 2 and 4 μM curcumin, which per se reduced cell proliferation without causing cell death, did not potentiate but instead slightly attenuated cisplatin-provoked apoptosis (Table 1).

**Mitochondria Regulatory Events.** Since ATO and lonidamine are mitochondria-targeting drugs, we analyzed the behaviour of proteins which are known to regulate apoptosis execution along the intrinsic (mitochondrial) pathway. Determinations were carried out at 16 h of treatment, the time at which apoptosis was firstly detected (see Fig. 1C). Some results, obtained by immunoblot assays, are represented in Fig. 4. It was observed that: (i) Treatments caused little effect on total Bcl-2 and Bax expression, except for a slight decrease in Bcl-2 in the curcumin plus lonidamine treatment. (ii) Nonetheless, treatments caused pro-apoptotic Bid and Bax activation, as manifested by the decrease in the amount of the 23 kDa Bid pro-form (as an indirect evidence of protein truncation/activation) and Bax translocation to the mitochondrial fraction (with concomitant decrease in the cytosolic fraction). (iii) Treatments stimulated cytochrome c release from mitochondria to the cytosol, required for apoptosome formation and activation, as demonstrated using cytosolic protein extracts. (iv) Treatments also down-regulated the expression of XIAP, which may relieve caspases from the inhibitory action exerted by this protein. (v) As a consequence, the treatments caused the truncation/activation of caspase-9, the initiator caspase in the mitochondrial pathway, and of the executioner caspase-3. In general, maximum alterations were produced by drug combinations (curcumin plus either lonidamine or ATO), which is consistent with the higher rate of apoptosis generation (see Figs. 1 and 3).
In addition, we analyzed the capacity of the treatments to cause mitochondrial membrane potential ($\Delta\Psi_m$) dissipation, as a manifestation of mitochondrial dysfunction. It was observed that curcumin plus ATO or lonidamine elicited the appearance of a subpopulation of cells with reduced $\Delta\Psi_m$, as indicated by the reduction in DilC1(5)-derived fluorescence (Fig. 5A). Nonetheless the importance of this event as a regulator of apoptosis was unclear. In fact, $\Delta\Psi_m$ dissipation was only detectable at 16 h of treatment (Fig. 5B), which coincided with the initiation of apoptosis execution (see Fig. 1C). Moreover, $\Delta\Psi_m$ reduction appeared as a caspase-dependent response, which was prevented by the pan-caspase inhibitor z-VAD-fmk (Fig. 5C, upper bar chart). In addition co-treatment with CsA (from 2 to 7 $\mu$M), an agent which inhibits mPTP by binding to cyclophylin D, failed to prevent $\Delta\Psi_m$ dissipation as well as apoptosis execution (Fig. 5C, and results not shown).

**ROS Production and Effect of Anti-oxidant Agents.** As commented above, ATO toxicity is increased under conditions of intracellular GSH depletion or ROS over-accumulation. Because of this, experiments were carried out to measure the capacity of curcumin to modulate intracellular GSH and ROS content. It was observed that 7.5 $\mu$M curcumin did not decrease and even slightly augmented GSH content, as measured by monochlorobimane derivatization. This contrasts with the depletion caused by the typical GSH synthesis inhibitor BSO, included as a control (Fig. 6A). On the other hand 7.5 $\mu$M curcumin caused an early and slight, but statistically significant increase in ROS accumulation, as measured using the anion superoxide-sensitive probe DHE (Fig. 6B). The importance of ROS production for curcumin-provoked apoptosis potentiation was proved using the ROS scavengers NAC and Mn-TBAP, a cell permeable superoxide dismutase mimetic agent. It was observed that co-treatment with the anti-oxidants greatly attenuated apoptosis generation (Fig. 6C), $\Delta\Psi_m$ dissipation (Fig. 6D) and cytochrome $c$ release (Fig. 6E) by curcumin plus either ATO or lonidamine. NAC and MnTABP also attenuated apoptosis generation by curcumin.
alone, when used at a toxic concentration (20 μM) (Fig. 6C). Finally, to corroborate the importance of the oxidant environment for apoptosis potentiation, cells were co-incubated with ATO or lonidamine plus a low concentration of H₂O₂, a paradigmatic pro-oxidant agent, or 2-ME, which is known to increase intracellular anion superoxide accumulation by inhibiting superoxide dismutase activity (Huang et al., 2000). The results in Fig. 6F indicated that H₂O₂ and 2-ME cooperate with ATO and lonidamine in more than additive manner to cause apoptosis, with better response for H₂O₂ in the case of ATO, and for 2-ME in the case of lonidamine. On the other hand, the pro-oxidant agents either did not affect cisplatin toxicity (2-ME) or caused less than additive effect (H₂O₂), a result which parallels the inability of curcumin to potentiate cisplatin-provoked apoptosis (see Fig. 3). Taken together these results indicate that ATO and lonidamine are ROS-sensitive drugs (i.e., its toxicity is increased under moderate pro-oxidant environment), and that curcumin-provoked ROS production may in part explain the capacity of the polyphenol to potentiate ATO- and lonidamine-provoked apoptosis.

Protein Kinase Modulation, and Effects of Protein Kinase Inhibitors. The MAPK p38 has been characterized as a kinase responsive to moderate oxidative stress (Kurata, 2000). In addition, other studies reported that curcumin stimulate ROS generation and JNK activation in osteoblast cells (Chan et al., 2006), potentiate ROS-dependent ERK activation and lethality in irradiated human cervical tumour cells (Javvadi et al., 2008), and cause ROS-mediated Akt inactivation in human renal cells (Woo et al., 2003). For these reasons, we wanted to measure the phosphorylation/activation state of these kinases in U937 cells treated curcumin, alone and in combination with ATO and lonidamine. The results were as follows (i) The treatments failed to significantly affect p38-MAPK and JNK phosphorylation (see supplemental Fig. 1). (ii) On the other hand curcumin, alone or with ATO, greatly stimulated ERK phosphorylation/activation at 14-24 h, and this response was
corroborated (24 h) using lonidamine plus curcumin (Fig. 7A). Nonetheless ERK activation did not explain apoptosis potentiation in our studies. In fact, the MEK/ERK inhibitors PD98059 (20 μM) or U0126 (2.5 μM), which successfully prevented kinase activation in U937 cells in preceding studies (Fernández et al., 2004), either did not modify (lonidamine plus curcumin) or augmented (ATO plus curcumin) apoptosis generation, as revealed by chromatin condensation/fragmentation (Fig. 7B) and cell cycle distribution (Fig. 7C). (iii) Finally curcumin, alone or with ATO, caused a slight decrease in Akt phosphorylation at 14-24 h, which was corroborated (24 h) in the combination of lonidamine plus curcumin (Fig. 8A). Noteworthy, co-treatment with the PI3K inhibitor LY294002 (30 μM), which greatly decreases Akt phosphorylation in U937 cells (Ramos et al., 2005), potentiated apoptosis induction by ATO alone, but not by lonidamine alone (Fig. 8B. left bar chart, and Fig. 8C). Nonetheless, co-treatment with LY294002 augmented the toxicity of curcumin alone, and as consequence also increased the toxicity of curcumin plus either ATO or lonidamine (Fig. 8B, right bar chart).

As complementary study we analyzed NF-κB activation, since the pro-apoptotic action of curcumin was frequently explained by the inhibition of this transcription factor (Lin, 2007; Reuter et al, 2008). Nonetheless, within the assayed time-periods (8 to 24 h), we did not detect significant alterations in p65-NF-κB translocation to the nucleus or in NF-κB binding to its DNA consensus sequence (see supplemental Fig. 2).
Discussion

The results in this work indicate that co-treatment with sub-cytotoxic curcumin concentrations greatly increases the apoptotic efficacy of ATO and lonidamine in U937 and HL60 AML cells and, albeit with lower intensity, K562 CML cells. To our knowledge this is first documented observation indicating ATO and lonidamine sensitization by curcumin, since as indicated above all preceding publications only reported protection by curcumin against arsenic toxicity. On the other hand, the cooperative response was negligible when the polyphenol was combined with cisplatin. There are several mechanisms which could explain the drug-dependent, differential efficacy of curcumin. One of them is the unequal sensitivity of ATO, lonidamine and cisplatin to the pro-oxidant environment, as discussed below. Another reason is that, while ATO and lonidamine exerted minimal effects on cell cycle distribution in U937 and HL60 cells, the DNA-damaging agent cisplatin delayed or blocked cell transition throughout S and G2 with concomitant decrease in the size of the G1 sub-population. This is important since cells at G2/M are more resistant to curcumin than cells traversing the G1 phase (Wang et al., 2008). In good agreement with this hypothesis, ATO caused G2/M arrest in K562 cells, and these cells were relatively resistant to apoptosis induction by ATO plus curcumin. Nonetheless other explanations are possible in this particular case, such as the high intrinsic resistance of K562 cells conferred by the p210Bcr/Abl kinase. Whatever the case, it must be noted that curcumin was reported to efficaciously potentiate cisplatin-provoked apoptosis in other cell types (Notarbolo et al., 2005; Chanvorachote et al., 2008), indicating that treatment effects are not only drug-specific but also cell type-specific.

In agreement with the properties of ATO and lonidamine as mitochondria-targeting drugs, the treatments activated apoptosis through the mitochondrial executioner pathway, as evidenced by Bax- and Bid-regulated cytochrome c release, down-
modulation of the caspase inhibitory XIAP protein, and the resulting activation of the caspase-9/caspase-3 pathway. In addition the treatments caused ΔΨm disruption, but the response pattern was not as initially expected. In fact, ΔΨm dissipation represented a late, caspase-dependent (z-VAD-inhibitable) event, occurring at same time as other apoptotic features (e.g., caspase-3 activation, chromatin condensation/fragmentation). Moreover, co-treatment with the mPTP inhibitor CsA, which is supposed to block mPTP opening, failed to prevent ΔΨm dissipation and apoptosis generation. Nonetheless these observations must be considered with caution, since drug responses are not necessarily equivalent when examined at the sub-cellular level (e.g., PTP opening in isolated mitochondria) and in entire cells, where multiple parameters may be affected. For instance, it was reported that CsA triggers ROS production in several cell models (Navarro-Antolin et al., 2007, and references therein), and ROS are determinants for ATO and lonidamine toxicity, as later commented. In addition the ATO concentration used by us (2 μM) is within the range of clinical useful concentrations, but is much lower than those currently used to analyze mPTP induction. Finally, lonidamine may inhibit mitochondria-bound hexokinase, affecting glucose metabolism in tumour cells (Fantin and Leder, 2006). Hence, its pro-apoptotic action might be mainly the consequence of ATP depletion instead of direct mPTP induction. These possibilities are currently under study.

In addition our results indicate that ATO as well as lonidamine are oxidant-sensitive agents, as proved by the increased toxicity when combined with low H2O2 concentrations or the superoxide dismutase inhibitor 2-ME. In spite of their normal protective, anti-oxidant action, acute exposure to some polyphenols may stimulate mitochondrial (Salvi et al., 2002) and extra-mitochondrial (Galati et al., 2002) ROS production. In this regard, Kikuchi et al. (2010) recently indicated that curcumin potentiated NADPH-derived anion superoxide generation by all-trans retinoic acid in U937 cells, although treatment with curcumin alone was ineffective. However, our
present results indicate that curcumin alone suffices to slightly stimulate anion superoxide generation in this cell line, although the exact mechanism was not investigated. ROS generation by a relatively high curcumin concentration (20 μM) might per se suffice to trigger apoptosis, while the slight ROS production caused by a lower (7.5 μM) concentration, being itself innocuous, may increase ATO and lonidamine toxicity, as proved by the protective action of the antioxidants NAC and MnTBAP. Moreover, as commented above, this mechanism may provide an alternative or complementary explanation for the inability of curcumin to potentiate cisplatin-provoked apoptosis. In fact, under the here used conditions cisplatin was little sensitive to the pro-oxidant environment, as revealed by co-treatment with H2O2 or 2-ME.

Finally, we analyzed the behaviour of some protein kinases the expression of which might undergo ROS-dependent alterations in curcumin-treated cells. Curcumin caused ERK phosphorylation/activation, but this response was not affected (curcumin plus lonidamine) or was even potentiated (curcumin plus ATO) by co-treatment with pharmacologic MEK/ERK inhibitors. This is consistent with the protective, anti-apoptotic action commonly attributed to this kinase, but contrasts with the pro-apoptotic function of curcumin-provoked ERK activation reported in another experimental model (Javvadi et al., 2008). In addition, in spite of its pro-oxidant action curcumin failed to significantly stimulate JNK and p38-MAPK activation. This later result was unexpected, since as earlier indicated p38-MAPK is considered as sensor of moderate oxidative stress, and this kinase was activated in a ROS-dependent manner by other phenolic agents in U937 cells (Sánchez et al., 2008). Nonetheless, it was recently reported that curcumin may cause p38-MAPK de-phosphorylation via MAP kinase phosphatase-5 (MAPK5) activation in prostate cells (Nonn et al., 2007). Thus, different opposite signals may probably concur to define the state of p38-MAPK activation in curcumin-treated cells. Finally, curcumin slightly caused Akt de-phosphorylation/inactivation. In accordance with its role as a protective kinase, Akt
inhibition may represent an additional mechanism explaining the potentiation by curcumin of ATO-provoked apoptosis, since ATO toxicity was also increased when combined with the PI3K inhibitor LY294002 instead of curcumin. The importance of Akt inhibition in the case of lonidamine is less clear, since LY294002 only augmented the toxicity of lonidamine in combination with curcumin (which was itself sensitive to the PI3K inhibitor), but not of lonidamine alone. Whatever the case, Akt down-regulation contrasts with the apparent inability of curcumin to affect basal NF-κB activity, as measured by p65 subunit translocation and transcription factor binding. This does not exclude that curcumin might inhibit NF-κB in other experimental conditions – e.g., treatment with curcumin alone for longer treatment periods, or curcumin in combination with stressing, NF-κB-activating agents, as we previously commented using another polyphenol (Sánchez et al., 2008).

Lonidamine is a well-tolerated drug, clinically employed against multiple types of solid tumours (Di Cosimo et al., 2008) and also of potential interest against some haematological malignancies (Solary et al., 2003). Nonetheless, as in the case of ATO the efficacy of this agent is normally low when used as monotherapy. The present results indicate that curcumin efficaciously cooperates with both ATO and lonidamine to induce apoptosis in acute myeloid leukaemia cell models, a response which, considering the high tolerability of this polyphenol, might be of therapeutic interest. Although other mechanisms are also possible, such cooperation may be in part explained by curcumin-provoked generation of moderate oxidative stress, and at least in the case of ATO also by Akt down-regulation.
References


Footnotes

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Y.S. and G.P.S. equally contributed to this work.

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Legends for Figures

**Fig. 1.** Apoptosis induction and cell cycle effects caused by ATO and curcumin in U937 cells. A, frequency of apoptotic cells, as determined by chromatin condensation/fragmentation, in untreated U937 cell cultures (Cont) and cultures treated with the indicated concentrations of ATO alone, curcumin (Cur) alone, and 2 μM ATO in combination with the indicated concentrations of curcumin (ATO+Cur). B, changes in cell viability, as determined by the MTT assay. The absorption values are represented in relation to the control, which received the arbitrary value of one. C, frequency of apoptotic cells at the indicated times of treatment. D, cell cycle distribution and frequency of apoptotic cells (Ap), as indicated by sub-G$_1$ DNA content. E, frequency of cells exhibiting phosphatidyl serine translocation, as measured by cell surface Annexin V binding (green fluorescence), and PI uptake (red fluorescence). A background of approximately 15% green fluorescent cells in curcumin-treated cultures was estimated to be due to drug auto-fluorescence (see Materials and Methods). In combined treatments, ATO and curcumin were simultaneously applied. Except when otherwise indicated, ATO was used at 2 μM and curcumin at 7.5 μM, and the measurements performed at 24 h of treatment. The results in A-C represent the mean ± SD of at least three determinations. The results in D and E are representative of one of two determinations.

**Fig. 2.** Apoptotic induction and cell cycle effects caused by ATO and curcumin in other leukaemia cell lines. A, frequency of apoptosis, as determined by chromatin condensation/fragmentation, in HL60 and K562 cell cultures treated with curcumin and the indicated ATO concentrations, alone and in combination. B, changes in viability in K562 cell cultures, as determined by the MTT assay. C, cell cycle distribution and frequency of apoptotic cells in K562 cell cultures treated with curcumin and 5 μM ATO. ATO was used at 2 μM in HL60 cells. Curcumin was used at 5 μM in HL60 cells, and...
at 10 μM in K562 cells. All determinations were carried out at 24 h of treatment. All other conditions were as in Fig. 1.

Fig. 3. Apoptosis induction and cell cycle effects caused by lonidamine, cisplatin and curcumin in U937 cells. A, frequency of apoptosis, as determined by chromatin condensation/fragmentation, in U937 cell cultures treated with the indicated concentrations of lonidamine (Lon, upper bar chart) and cisplatin (CDDP, lower bar chart), alone and in combination with curcumin. B, changes in cell viability, as determined by the MTT assay. C, cell cycle distribution and frequency of apoptotic cells. D, frequency of cells exhibiting phosphatidyl serine translocation, as measured by cell surface Annexin V binding (green fluorescence), and PI uptake (red fluorescence). Except when otherwise indicated, curcumin was used at 7.5 μM, Lon at 50 μM and CDDP at 4 μM. All determinations were carried out at 24 h of treatment. All other conditions were as in Fig. 1.

Fig. 4. Expression of apoptosis-regulatory proteins. U937 cells were treated for 16 h with curcumin (7.5 μM), ATO (2 μM) and lonidamine (100 μM), alone and in combination. The figure shows the relative levels of Bcl-2, Bid (21 kDa pro-form) and XIAP, as determined by immunoblot in total cellular extracts; the release of cytochrome c to the cytosol, as determined by the increase in cytosolic extracts; Bax translocation to mitochondria, as determined by the increase in mitochondrial fractions and decrease in cytosolic extracts; and pro-caspase-9 and -3 maturation, as revealed by the appearance of cleavage-derived fragments. The numbers below each blot indicate the intensity of bands in relation to the corresponding control (which received the arbitrary value of 1), as determined by densitometry. The total levels of β-actin were measured as a loading control. All other conditions were as in Fig. 1.
Fig. 5. Dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$), and effects of caspase and mPTP inhibitor. A,B, U937 cells were treated for the indicated time periods with 2 $\mu$M ATO or 100 $\mu$M lonidamine, alone (-) or in combination with 7.5 $\mu$M curcumin (+ Cur), and the alterations in $\Delta\Psi_m$ were determined by DilC1(5) staining and flow cytometry. Note the appearance of a subpopulation of cells with decreased fluorescence (cells with low $\Delta\Psi_m$), clearly detected at 16 h in the combined treatments. C, Frequency of cells with low $\Delta\Psi_m$ (upper bar chart), and frequency of apoptotic cells (lower bar chart), upon treatment for 16 h with ATO plus curcumin or ATO plus lonidamine, either in the absence (-) or the presence of 50 $\mu$M z-VAD-fmk (+ z-VAD) or 5 $\mu$M CsA (+ CsA). The inhibitors were applied 2 h before the other treatments. All other conditions were as in Fig. 1.

Fig. 6. Changes in intracellular GSH and ROS levels, and effect of anti-oxidants or pro-oxidants. A, changes in intracellular GSH concentration, as indicated by monochlorobimane derivatization, in cells treated for the indicated time-periods with curcumin or 1 mM BSO, included as a control. The results are represented in relation to untreated (Cont) cells (approximate GSH content, 9.5 nmol/10$^6$ cells), which received the arbitrary value of one. B, intracellular ROS accumulation, as indicated by DHE-derived fluorescence, in U937 cells treated for the indicated time-periods with curcumin, ATO and lonidamine. The results are represented in relation to the untreated cells, which received the arbitrary value of one. Asterisks indicate significant differences in relation to control. An example of cell distribution in untreated- (Cont) and curcumin-treated cultures is indicated at the bottom. The vertical dotted line indicates the main value in control, to better discern the displacement caused by curcumin. C-E, frequency of apoptotic cells (C), $\Delta\Psi_m$ dissipation (D), and cytochrome c release to the cytosol (E) upon treatment with 20 $\mu$M curcumin alone, or with the combinations of ATO plus curcumin or lonidamine plus curcumin, in the absence (-) or
the presence of NAC (+ NAC) or MnTBAP (+ MnTBAP). F, frequency of apoptosis upon treatment with ATO, lonidamine, and cisplatin, alone (-) or together with H₂O₂ (+ H₂O₂) or 2-methoxyestradiol (+ 2-ME). Symbols indicate that the values in the combined treatments (ATO + H₂O₂, Lon + H₂O₂, ATO + 2-ME, Lon + 2-ME) is equivalent (#) or significantly higher (asterisks) than the sum of values in the corresponding single treatments. Curcumin was used at 7.5 μM (except when otherwise indicated), ATO at 2 μM, lonidamine at 100 μM, cisplatin at 4 μM, NAC at 10 mM, MnTBAP at 100 μM, H₂O₂ at 40 μM, and 2-ME at 0.5 μM. NAC and MnTBAP were applied 2 h before the other agents. Results in D and E were obtained at 16 h of treatment, and in C and F at 24 h of treatment. All other conditions were as in Fig. 1 and 3.

**Fig. 7.** ERK modulation and effect of MEK/ERK inhibitors. A, relative levels of total (T) and phosphorylated (P) ERK, upon treatment for the indicated time-periods with curcumin, ATO plus curcumin, or ATO plus lonidamine, as determined by immunoblot assays. B, frequency of apoptotic cells at 24 h of treatment with ATO or lonidamine alone (-), with ATO or lonidamine plus curcumin (+Cur), and with ATO or lonidamine plus curcumin in the presence of the MEK/ERK inhibitors PD98059 (+PD+Cur) or U0126 (+U+Cur). Asterisks indicate significant differences in relation to control. C, cell cycle distribution and frequency of apoptotic (sub-G₁) cells at 24 h of treatment with the indicated agents. The MEK/ERK inhibitors were applied 1 h in advance to the other agents. Curcumin was used at 7.5 μM, ATO at 2 μM, PD98049 at 30 μM, and U0126 at 2.5 μM. All other conditions were as in Fig. 1.

**Fig. 8.** Akt modulation and effects of PI3K inhibitor. A, relative levels of total (T) and phosphorylated (P) Akt, upon treatment for the indicated time-periods with curcumin, ATO plus curcumin, or ATO plus lonidamine, as determined by immunoblot assays
The numbers below the Akt-P blot indicate the intensity of bands in relation to the control (which received the arbitrary value of 1), as determined by densitometry. B, frequency of apoptotic cells at 24 h of treatment with the PI3K inhibitor LY294002 (LY), and with ATO or lonidamine, either alone (-) or in combination with LY294002 (+LY) (left bar chart); or with curcumin, ATO plus curcumin and lonidamine plus curcumin, either alone (+Cur) or in combination with LY294002 (+Cur+LY) (right bar chart). Asterisks indicate significant differences in relation to control. C, cell cycle distribution and frequency of apoptotic (sub-G₁) cells at 24 h of treatment with the indicated agents. ATO was used at 2 μM, curcumin at 7.5 μM, and LY294002 at 30 μM. LY294002 was applied 1 h in advance to the other drugs. All other conditions were as in Fig. 1.
TABLE 1

Modulation of cisplatin-provoked toxicity and G2/M arrest by curcumin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number (%) of Cont</th>
<th>Apoptotic cells (%)</th>
<th>Cells at G2/M (%)</th>
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<tr>
<td>Cont</td>
<td>100</td>
<td>4±1</td>
<td>25±3</td>
</tr>
<tr>
<td>Cur 2 μM</td>
<td>85±9</td>
<td>5±2</td>
<td>23±4</td>
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<tr>
<td>Cur 4 μM</td>
<td>65±6</td>
<td>7±2</td>
<td>25±4</td>
</tr>
<tr>
<td>CDDP 2 μM</td>
<td>15±3</td>
<td>43±6</td>
<td>44±5</td>
</tr>
<tr>
<td>CDDP 2 μM + Cur 2 μM</td>
<td>17±2</td>
<td>28±4</td>
<td>42±6</td>
</tr>
<tr>
<td>CDDP 2 μM + Cur 4 μM</td>
<td>15±5</td>
<td>35±6</td>
<td>45±7</td>
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</table>

All determinations were carried out at 72 h of treatment. Values represent the mean ± SD of three assays.

* Cells were seeded at 10^5 cells/ml. The approximate final concentration in Cont was 17x10^5 cells/ml.

* Measured by chromatin condensation/fragmentation.

* Relative to the total number of cells at G1 + S + G2/M (excluding cells at sub-G1).
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

A

B

C

Cells with low $\Delta\Psi_m$ (%) vs. time (h)

Cont 16 h
Cur 16 h
ATO 16 h
Lon (100 $\mu$M) 6 16

Cells with low $\Delta\Psi_m$ (%) vs. time (h)

Cont z-VAD CsA Cur+ATO Cur+Lon (100 $\mu$M)

Apoptotic cells (%) vs. time (h)

Cont z-VAD CsA Cur+ATO Cur+Lon (100 $\mu$M)
Fig. 6
### Fig. 7

#### A

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
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<tr>
<td>Cont</td>
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<tr>
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</tr>
<tr>
<td>Lon50 +Cur</td>
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</tr>
<tr>
<td>Lon100 +Cur</td>
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#### B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (%)</th>
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</thead>
<tbody>
<tr>
<td>Cont</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>U</td>
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</tr>
<tr>
<td>+PD +Cur</td>
<td>*</td>
</tr>
<tr>
<td>+Cur</td>
<td>*</td>
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</table>

#### C

- **Cell number**
  - Cont: 193
  - ATO + Cur: 1118
  - Lon50 + Cur: 146

- **PI-derived fluorescence**
  - PD: 198
  - PD + ATO + Cur: 182
  - PD + Lon50 + Cur: Ap
Fig. 8