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Letter to the Editor

Quassinoids can induce mitochondrial membrane depolarisation and caspase 3 activation in human cells

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Dear Editor,

Natural products have long been a source of cure for cancer, and more than 1000 species of plants have been found to possess significant antineoplastic properties. Among these there are *Ailanthus* species, whose extracts have long been traditionally used as antitumoral in China and other Asian countries.[1–3] From *Ailanthus excelsa* we isolated a purified quassinoid, Picrasa-3,13(21)-diene-2,16-dione, 11,20-epoxy-1,11,12-trihydroxy,(1β,11β,12α)-(9Cl) (ailanthone), that induced mitochondrial membrane depolarisation and caspase 3 activation in Jurkat cells. Interestingly, suboptimal doses of the compound significantly synergised with recombinant TRAIL in inducing apoptosis. This information can contribute to explain the antineoplastic properties of *Ailanthus* and indicates that quassinoids can allow further investigation for a possible use in therapy.

Dried roots (200 g) were extracted with methanol and fractionated by gel-permeation chromatography on a Sephadex LH-20 column, eluting with MeOH. In total, 126 of about 10 ml each were obtained and pooled in 20 main fractions on the basis of their TLC similarity in BuOH : AcOH : H_2O (12 : 3 : 5) and $CHCl_3$: MeOH : H_2O (70 : 30 : 3). Fractions 10, the most active in the bioassays, was purified by RP-HPLC on a C18 μ-Bondapack column (30 cm $\times$ 7.8 mm), eluting with a mixture of H_2O–MeOH 40 : 60, and pure ailanthone (32 mg) was recovered. Structural determination of this compound was performed by analyses of ^{1}H, ^{13}C NMR and ^{13}C NMR DEPT data. Ailanthione was diluted in 10% FCS-RPMI and added to Jurkat cell cultures at the indicated concentrations (Figure 1Aa) and for the indicated times (Figure 1Ab); then apoptosis was evaluated by incorporation of propidium iodide in the DNA of permeabilised cells.[4] Ailanthone induced the appearance of $>$50% hypodiploid cells in cell cultures: its maximal effect was exerted at a concentration of 1 μg/ml and 72 h following its addition to cultures (Figure 1A).

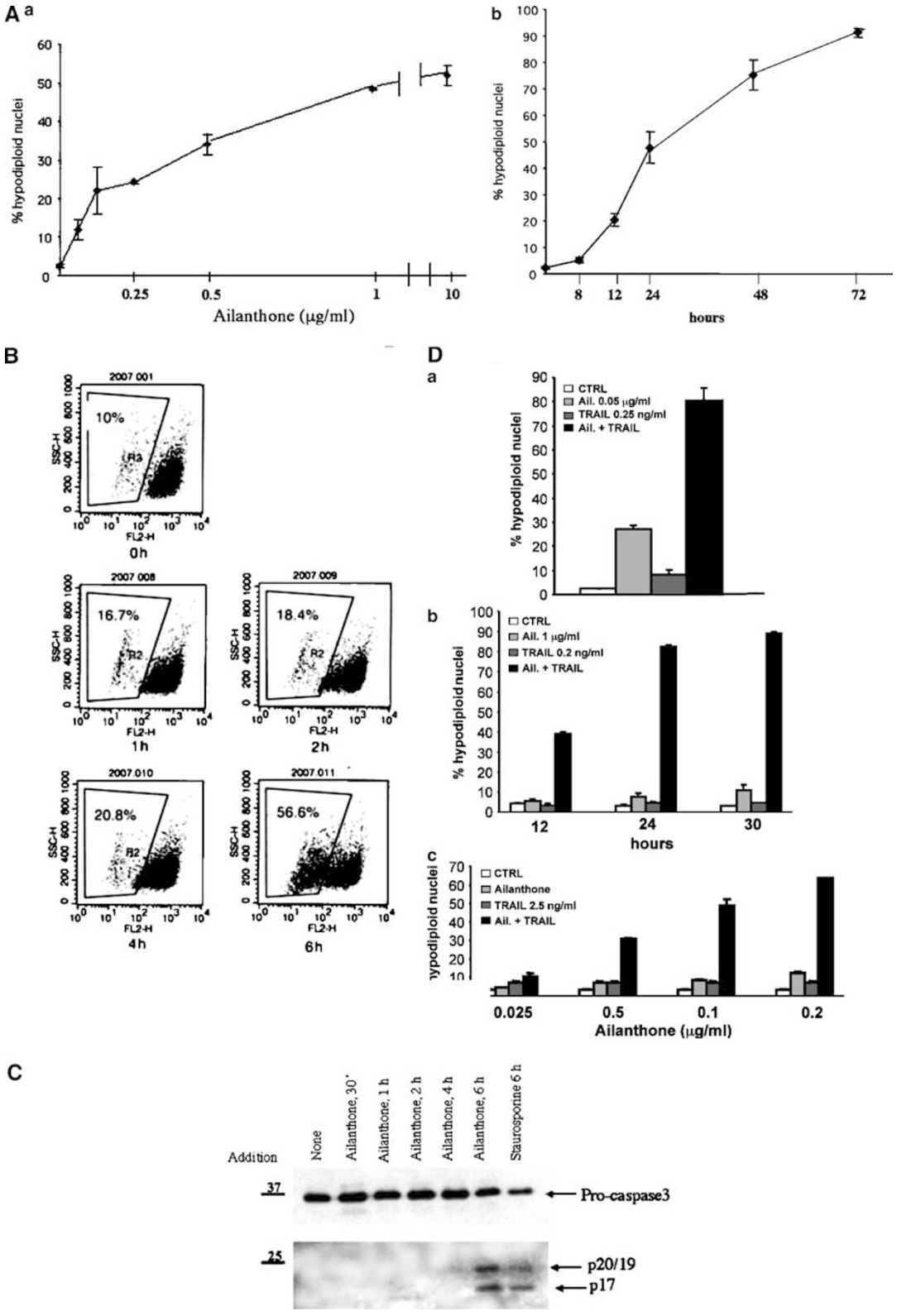
Few hours following its administration to the cells, ailanthone induced mitochondrial membrane depolarisation, examined using the probe tetramethylrhodamine ethyl ester (TMRE)[5] (Figure 1B), and subsequently activation of caspase 3, evident in Western blot (Figure 1C). Since quassinoids are able to interact with cell membranes,[6] ailanthone might trigger apoptosis by directly altering mitochondrial membrane permeability. However, an indirect effect, mediated by ailanthone interaction with plasma membrane NADH oxidase and modification of the intracellular redox state, cannot be excluded – although we did not observe any significant modification in reactive oxygen species (ROS) concentration following cell exposure to the molecule (results not shown).

These findings indicated that ailanthone could induce apoptosis in leukemic cells at clinically achievable concentrations. We verified whether the molecule could also synergise with recombinant TRAIL.[7] As illustrated in Figure 1Da, cell apoptotic response ($>$80% of hypodiploidy) was elicited in Jurkat cells by the combined addition of suboptimal concentrations of ailanthone and recombinant TRAIL, which separately induced $<$30 and 10%, respectively, of hypodiploidy. The two compounds displayed a similar synergy also in cells of the thyroid papillary carcinoma line NPA. Indeed, after a 12 h incubation with suboptimal concentrations of the two molecules, NPA cells displayed about 40% of apoptosis, while cultures with ailanthone or TRAIL alone showed $<$10% of apoptotic cells. Synergy was even more evident at 24 and 30 h, when $>$80% apoptosis was reached in cells cultured with the combined molecules (Figure 1Db). Finally, to further verify the ailanthone-TRAIL synergy in a third different cell line, we analysed the effect of the two compounds on

Figure 1 (**A**) Induction of Jurkat cell apoptosis by ailanthone. a – Cells (1×10^6/ml) were incubated in 10% FCS-RPMI medium, in the presence of the indicated concentrations of ailanthone, for 24 h. Then, hypodiplody was analysed by propidium iodide incorporation in permabilised cells and flow cytometry.[4] b – Cells (1×10^6/ml) were incubated in 10% FCS-RPMI medium, in the presence of 0.13 μg/ml of ailanthone, for the indicated times, and then hypodiploidy was evaluated. (**B**) – Induction of mitochondrial membrane depolarization by ailanthone. Cells (1×10^6/ml) were incubated in 10% FCS-RPMI medium, in the presence of 0.13 μg/ml of ailanthone, for the indicated times. Then, the cells were incubated with TMRE at 37°C for 10 min in the dark and analysed by flow cytometry.[5] (**C**) – Induction of caspase 3 activation by ailanthone. Cells were plated at a density of 1×10^6 cells/ml in 10% FCS-RPMI medium, in the presence or absence of ailanthone (0.13 μg/ml) or staurosporine (1.5 μM). Cells were collected, washed twice in PBS, lysated using100 μl of Laemnlie buffer 1 $\times$ (Tris HCl pH 6.8 0, 25 M, SDS 2.5%, glycerol 2.5%, 2-mercaptoethanol 5%, Bromophenol blue) and sonicated three times at 50% amplitude. Proteins were separated by SDS-polyacrilamide gel followed by immunoblotting with polyclonal anti-caspase3 (StressGen, Victoria BC, Canada), incubated with horseradish peroxidase-conjugated secondary antibody and detection using enhanced chemiluminescence technology following standard protocols. (**D**) – Synergy of ailanthone and TRAIL in inducing cell apoptosis. Jurkat (a), NPA (b) or SaOs-2 (c) cells were incubated in 10% FCS-RPMI medium, in the presence of the indicated concentrations of ailanthone and/or recombinant TRAIL (Alexis Biochemicals, San Diego, CA, USA), for 24 h (a, c) or the indicated times (b), and then hypodiploidy was evaluated

osteosarcoma SaOs-2 cells. Figure 1Dc shows the dose-dependent synergistic effect of ailanthone with a suboptimal concentration of TRAIL in these cells.

These results suggest that low concentrations of ailanthone might help reducing TRAIL doses in systemic administration and contribute to indicate quassinoids as candidates for



in vivo analyses of their value in the management of human malignancies.[3,8]

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