Mode of action study on LTX-315-induced cell death

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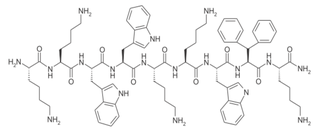
Introduction

LTX-315 has been developed as an amphipathic cationic peptide that kills cancer cells. Firstly, we investigated the question whether LTX-315 induces apoptosis or necrosis. Transmission electron microscopy or morphometric analysis of chromatin-stained tumor cells revealed that LTX-315 failed to induce apoptotic nuclear condensation and rather induced a necrotic phenotype. Accordingly, LTX-315 failed to stimulate the activation of caspase-3, and inhibition of caspases by means of Z-VAD-fmk was unable to reduce cell killing by LTX-315. In addition, two prominent inhibitors of regulated necrosis (necroptosis), namely, necrostatin-1 and cycosporin A, failed to reduce LTX-315-induced cell death. In conclusion, it appears that LTX-315 triggers unregulated necrosis, which may contribute to its pro-inflammatory and pro-immune effects. Secondly, we investigated the putative involvement of mitochondria in the cytotoxic action of LTX-315. Subcellular fractionation of LTX-315-treated cells, followed by mass spectrometric quantification, revealed that the agent was enriched in mitochondria. LTX-315 caused an immediate arrest of mitochondrial respiration without any major uncoupling effect. Accordingly, LTX-315 disrupted the mitochondrial network, dissipated the mitochondrial inner transmembrane potential, and caused the release of mitochondrial intermembrane proteins into the cytosol. LTX-315 was relatively inefficient in stimulating mitophagy. Cells lacking the two pro-apoptotic multidomain proteins from the BCL-2 family, BAX and BAK, were less susceptible to LTX-315-mediated killing. Moreover, cells engineered to lose their mitochondria (by transfection with Parkin combined with treatment with a protonophore causing mitophagy) were relatively resistant against LTX-315, underscoring the importance of this organelle for LTX-315-mediated cytotoxicity Altogether, these results support the notion that LTX-315 kills cancer cells by virtue of its capacity to permeabilize mitochondrial membranes. Thirdly, based on the observation that intratumorally injected LTX-315 stimulates a strong T lymphocyte-mediated anticancer immune response, we investigated whether LTX-315 may elicit the hallmarks of immunogenic cell death (ICD). Using a panel of biosensor cell lines and robotized fluorescence microscopy coupled to automatic image analysis, we observed that LTX-315 induces all known ICD characteristics. This conclusion was validated by several independent methods including immunofluorescence stainings (for calreticulin), bioluminescence assays (for ATP), immunoassays (for HMGB1) and RT-PCRs (for type-1 interferon induction). When injected into established cancers, LTX-315 caused a transiently hemorrhagic focal necrosis that was accompanied by massive release of HMGB1 (from close-to-all cancer cells), as well caspase-3 activation in a fraction of the cells. LTX-315 was at least as efficient as the positive control, the anthracycline mitoxantrone, in inducing local inflammation with infiltration by myeloid cells and T lymphocytes. Collectively, these results support the idea that LTX-315 can induce ICD, hence explaining its capacity to mediate immune-dependent therapeutic effects.

Results

1) New anticancer agent LTX-315

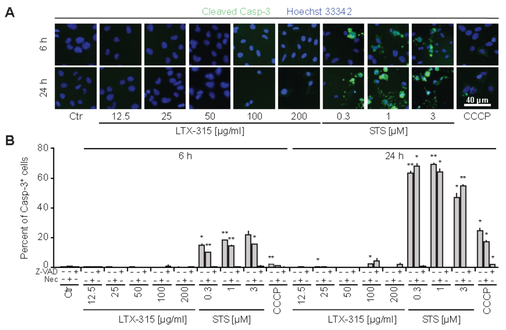


Chemical structure of LTX-315
MW:1439.82

Lytix Biopharma is developing a new anticancer drug candidate, an antimicrobial peptide typically has a cationic amphiphilic structure

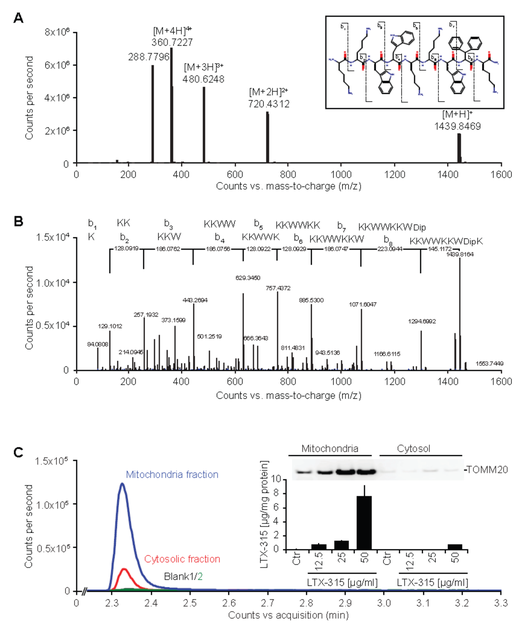
2) Failure of LTX-315 to induce hallmarks of apoptosis

Ultrastructual characteristics of LTX315-induced cell death. Note the presence of dilated mitochondria in cells treated with 12.5 or 50 µM of LTX-315.

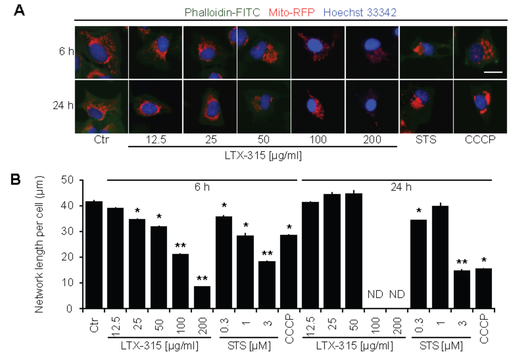


Failure of LTX-315 to induce caspase-3 activation and nuclear shrinkage. Representative images are shown in A. Quantitative results (means ± SD of triplicates) are shown in B. The frequency of Casp3a+ cells is shown for each treatment, cells with normal morphology (not shrunken) is displayed. Asterisks indicate significant differences with respect to untreated controls.

3) Mitochondrial enrichment and effects of LTX-315

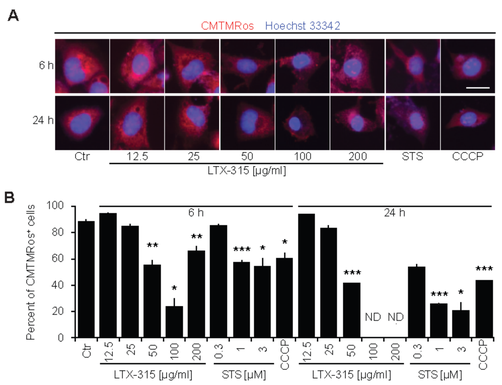


Mass spectrometric detection of LTX-315 enriched in the mitochondrial fraction. Each fraction was analyzed and yielded in chromatographic peaks of the LTX-315 in the mitochondria and cytosolic fractions with different amplitudes. Subsequently the concentration of LTX-315 peptide was evaluated by BSA protein quantification in each fraction.

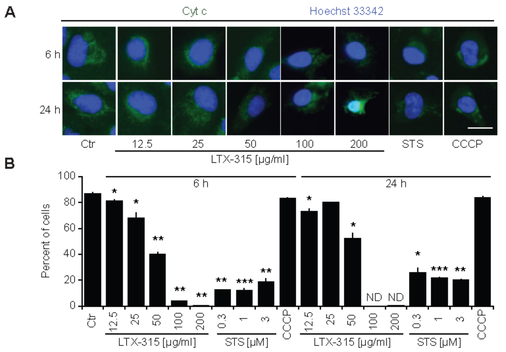


Functional and morphological disruption of mitochondria by LTX-315. Results are means ± SD of triplicates. Asterisks indicate significant (unpaired Student t test) changes with respect to untreated controls (Ctr).
*** p<0.05; ** p<0.01**

4) Mitochondrial permeabilization by LTX-315

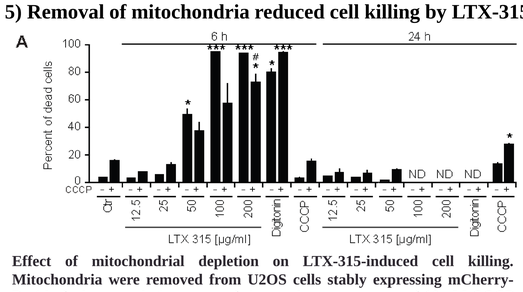


Dissipation of the mitochondrial transmembrane potential (Dy_m)



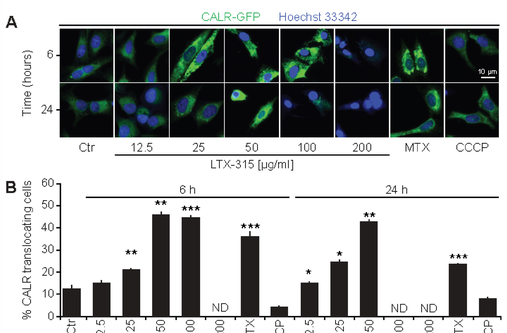
Mitochondrial outer membrane permeabilization induced by LTX-315.

5) Removal of mitochondria reduced cell killing by LTX-315



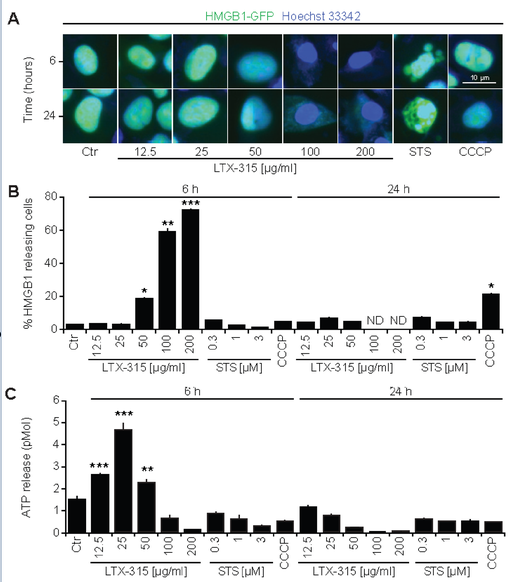
Effect of mitochondrial depletion on LTX-315-induced cell killing. Mitochondria were removed from U2OS cells stably expressing mCherry-Parkin fusion protein by means of the continuous presence of 10 µM CCCP for 48 h. Subsequently the cells were treated with the indicated concentrations of LTX-315, STS or CCCP for additional 6 or 24 h. Following the cells were subjected to microscopical analysis and the percent of cells that depict necrotic phenotypes are shown.

6) LTX-315 induces calreticulin exposure



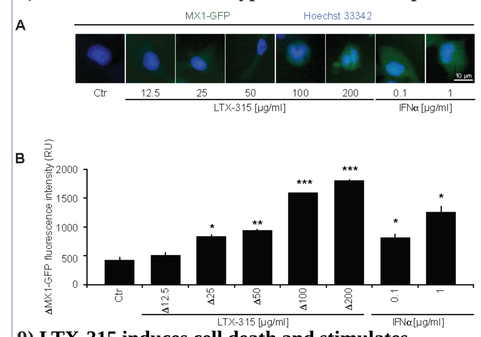
U2OS stably expressing a calreticulin-GFP fusion protein were treated with the indicated agents, fixed at the indicated time points, counterstained with the chromatin dye Hoechst 33342 and subjected to automatic fluorescence microscopy

7) LTX-315 induces the release of HMGB1 and ATP

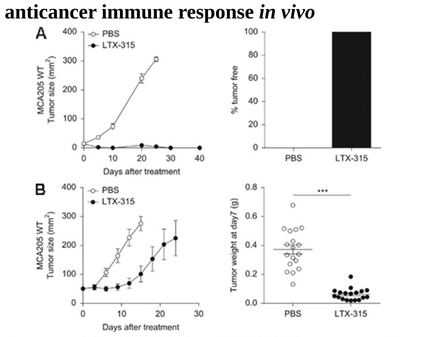


Release of ATP from cells exposed to LTX-315 and release of nuclear HMGB1 from cells exposed to LTX-315. Asterisks indicate significant differences (unpaired Student t test) with respect to untreated controls.
***p<0.05; **p<0.01; ***p<0.001.**

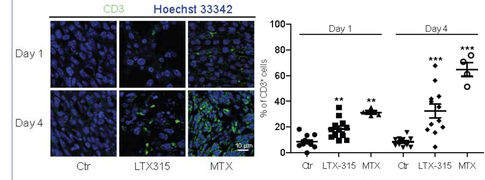
8) LTX-315 stimulates a type-1 interferon response



9) LTX-315 induces cell death and stimulates anticancer immune response *in vivo*



Anticancer activity of LTX-315 on MCA205 fibrosarcomas. Tumor growth kinetics (left) and weight at killing (right) are depicted.



MCA205 fibrosarcomas were injected locally with PBS (control, Ctr), LTX-315, or mitoxantrone (MTX).

Conclusion

The present data indicate that LTX-315 appears to activate a direct pronecrotic pathway and preferentially enriches at mitochondrial membranes, causing their permeabilization, meaning that the barrier function of both the inner and the outer mitochondrial membrane is lost. LTX-315 can also induce all hallmarks of ICD including CALR exposure, ATP release, HMGB1 exodus and type-1 IFN responses.