Short Communication

Trypanosoma brucei: inhibition of cathepsin L is sufficient to kill bloodstream forms

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A B S T R A C T

The lysosomal cysteine protease activity of *Trypanosoma brucei* comprises a cathepsin B enzyme (*Tb*CATB) and a cathepsin L enzyme (*Tb*CATL). Inhibition of the cysteine protease activity is lethal to bloodstream-form trypanosomes but it was not entirely clear which of the two enzymes are essential for survival of the parasites. Here we show that the vinyl sulfone compound LU-102 selectively inhibits *Tb*CATL without affecting *Tb*CATB and the proteasomal trypsin-like activity within trypanosomes. Therefore, the trypanocidal activity displayed by LU-102 can be attributed solely to the inhibition of *Tb*CATL demonstrating that this enzyme is essential to the survival of *T. brucei*.

Keywords:
Trypanosoma brucei
African trypanosomiasis
Cysteine protease
Protease inhibitor

Bloodstream forms of *Trypanosoma brucei* express two cathepsin-like cysteine proteases, cathepsin B (*Tb*CATB) and cathepsin L (*Tb*CATL) [1,2]. With respect to the three subspecies of *T. brucei*, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*, the enzymes show high sequence identity to each other (*Tb*CATL: >98%; *Tb*CATB: >99%). The two enzymes are localised to the lysosomal compartment and are involved in the degradation of endocytosed host proteins [3,4]. *Tb*CATL is responsible for the majority of the cysteine protease activity [5]. Both proteases have been implicated to be essential for the survival of the parasite [3,4]. RNA interference data indicated the essentiality of *Tb*CATB despite only a modest reduction of 32% of the *Tb*CATB protein [4] while chemical target validation studies using small-molecule cysteine proteases inhibitors pointed towards *Tb*CATL as the vital enzyme [6]. Using the newly developed compound LU-102 (Fig. 1), we show here that inhibition of *Tb*CATL alone is sufficient to kill bloodstream forms of *T. brucei in vitro*.

The compound LU-102 is a peptidyl vinyl sulfone and was initially developed as an inhibitor of the trypsin-like activity of the mammalian proteasome [7]. By contrast, LU-102 was recently shown not to inhibit the trypsin-like activity of the proteasome of *T. brucei* [8]. The observed trypanocidal activity of LU-102 was subsequently attributed to the activity of the compound to inhibit also cathepsins [7,8]. Other targets could be excluded because peptidyl vinyl sulfones react exclusively with active site cysteine sulfhydryl and threonine hydroxyl; they do not react with the serine hydroxyl of serine proteases [9].

First, the inhibitory selectivity of LU-102 for *Tb*CATB and *Tb*CATL was determined using *T. brucei* cell extracts with the fluorogenic substrates Z-RR-AMC (benzyloxycarbonyl-arginyl-arginyl-7-amido-4-methyl coumarin) and Z-FR-AMC (benzyloxycarbonyl-phenylalanyl-arginyl-7-amido-4-methyl coumarin). Whereas Z-RR-AMC is cleaved by *Tb*CATB but not by *Tb*CATL, both *Tb*CATB and *Tb*CATL hydrolyse Z-FR-AMC [10,11]. LU-102 showed a dose-dependent inhibitory effect on the Z-FR-AMC hydrolysing activity with a half-maximal inhibitory concentration (IC_{50}) of 0.39 μM (Fig. 2A). As hydrolysis of Z-FR-AMC is mainly due to the activity of *Tb*CATL, this finding indicates that LU-102 is an inhibitor of cathepsin L. On the other hand, the Z-RR-AMC hydrolysing activity was unaffected by LU-102 (Fig. 2A). As Z-RR-AMC is only cleaved by *Tb*CATB, this result suggests that LU-102 does not inhibit this enzyme. The remaining Z-FR-AMC hydrolysing activity observed at 100 μM LU-102 (14.4% of control) was probably due to uninhibited *Tb*CATB activity and/or to *Tb*CATL that was not completely inhibited.

Next, the trypanocidal activity of LU-102 was determined with bloodstream forms of *T. brucei* using the resazurin (Alamar blue) assay described previously [12]. LU-102 showed a dose-dependent effect on the growth of trypanosomes with a MIC (minimum inhibitory concentration, i.e., that concentration of a compound at which all cells were killed) value of 25 μM and a GI_{50} (50% growth inhibition, i.e., that concentration of a compound necessary to reduce the growth rate of cells by 50% to that of controls) value 10.2 μM (Fig 2B). Whereas a MIC value was not determined previously, the GI_{50} value was comparable to that recently published (6.9 μM [8]). Based on the findings that LU-102 does not inhibit *Tb*CATB (see above) and the trypsin-like activity of the trypanosomal proteasome [8], the trypanocidal activity of the compound seems to be solely due to inhibition of *Tb*CATL, indicating that blocking the activity of *Tb*CATL is sufficient to kill bloodstream forms of *T. brucei*.

In order to prove that the trypanocidal activity of LU-102 is indeed only due to inhibition of *Tb*CATL, bloodstream forms of *T. brucei* were incubated with the compound for 2 h at the lethal concentration of 25 μM (MIC value; see Fig. 2B) and the residual peptidase activity in cell extracts was measured using Z-FR-AMC and Z-RR-AMC. The Z-FR-AMC hydrolysing activity was inhibited by 95.0% after incubating trypanosomes for 2 h with 25 μM LU-102 (Fig. 3A). The remaining hydrolytic activity of 5.0% was most likely due to *Tb*CATB as the Z-FR-AMC hydrolysing activity was almost completely abolished (by 99.6%) in parasites incubated with 25 μM of the non-selective cysteine protease inhibitor Z-Phe-Ala-diazomethylketone (Z-FA-DMK) for 2 h (Fig. 3B). On the other hand, the Z-RR-AMC hydrolysing activity was only inhibited by 27.0% after exposing the parasites for 2 h to 25 μM LU-102 (Fig. 3A). However, Z-RR-AMC hydrolysing activity in trypanosomes treated with LU-102 was not statistically significantly different from that of control parasites (Fig. 3A). In contrast, treatment of trypanosomes with 25 μM Z-FA-DMK for 2 h resulted in 94.6% inhibition of the Z-RR-AMC hydrolysing activity (Fig. 3B). These findings confirm the suggestion that LU-102 is not a potent inhibitor of *Tb*CATB. In addition, an inhibition of

*Tb*CATB by 27.0% would certainly not be sufficient to kill bloodstream forms of *T. brucei*. For example, the CATB-specific inhibitor CA-074 was shown to inhibit *Tb*CATB in trypanosomes by 95% after incubating the parasites with 100 µM of the compound for 2 h, yet CA-074 displayed no trypanocidal activity [6].

There was a slight difference in the inhibitory potency of LU-102 when using cell extract and live trypanosomes. Whereas 100 µM LU-102 inhibited the Z-FR-AMC hydrolysing activity by 85.6% in cell extract, 25 µM of the compound caused 95.0% inhibition of this peptidolytic activity in live trypanosomes (compare Fig 2A with Fig. 3A). Likewise, 100 µM LU-102 blocked the Z-RR-AMC hydrolysing activity by only 4.2% in cell extract, whereas 25 µM of the compound inhibited this peptidolytic activity by 27.0% in live trypanosomes (compare Fig 2A with Fig. 3B). However, it has been shown that many cysteine protease inhibitors suppress more efficiently *Tb*CATL and *Tb*CATB activity within trypanosomes than in trypanosome cell lysates [6]. The reason for this is that the intralysosomal milieu is a reducing environment [13-15] which facilitates the inactivation reaction of cysteine protease inhibitors with the active site cysteine residue. This suggestion was confirmed by demonstrating that cathepsin enzymes are more efficiently inhibited by cysteine protease inhibitors in the presence of thiols (dithiothreitol (DTT) and glutathione) [5,6]. To determine whether a reducing environment enhances the inhibitory potency of LU-102, the effect of the reducing agent DTT on the inhibition of the Z-FR-AMC and Z-RR-AMC hydrolysing activity by LU-102 in cell extracts was investigated. In the absence of DTT, pre-incubation of cell extracts with 25 µM of LU-102 inhibited the hydrolysis of Z-FR-AMC by 69.9% (Fig 3C). In contrast, in the presence of 2.5 mM DTT, pre-treatment of cell extracts with 25 µM LU-102 lead to 94.8% inhibition (Fig. 3C), which was almost identical to the extent of inhibition observed in live trypanosomes (95.0%; see Fig. 3A). Likewise, pre-treatment of cell extract with the compound in the absence of DTT resulted in the inhibition of the Z-RR-AMC hydrolytic activity by just 7.4% while in the presence of the thiol the hydrolysis of the peptide was inhibited by 21.7% (Fig. 3D). The inhibition in presence of DTT was similar to that observed for LU-102 in live trypanosomes (27.0%; see Fig. 3B). However, as for live trypanosomes, the inhibition of the Z-RR-AMC hydrolysing activity by LU-102 in cell extracts in the presence of DTT was not statistically significantly different from that of the DMSO control (Fig. 3B). These findings confirm that the inhibition reaction of LU-102 with the active site cysteine residue of *Tb*CATB and *Tb*CATL is enhanced in a reducing environment.

Previously it was shown that inhibition of lysosomal cysteine proteases in bloodstream-form trypanosomes is associated with the accumulation of transferrin in the lysosome [16,17]. Further analysis revealed that only blockage of *Tb*CATL resulted in considerable accumulation of transferrin in the lysosome while inhibition of *Tb*CATB did not interfere with the degradation of the iron-transport protein [6]. To determine whether treatment of trypanosomes with LU-102 leads to accumulation of transferrin within the lysosome, parasites were incubated with fluorescein-labelled transferrin in the presence of 25 μM LU-102 or Z-FA-DMK for 2 h. Treatment of trypanosomes with 25 μM LU-102 led to the accumulation of transferrin (Fig. 3E). Based on the median of the fluorescence intensity signal, LU-102 treated trypanosomes accumulated 4.7-times more transferrin than control cells treated with DMSO alone (Fig. 3E). However, trypanosomes incubated with 25 μM Z-FA-DMK accumulated about twice more transferrin than LU-102 treated cells (Fig. 3E). This result showed that inhibition of *Tb*CATL by LU-102 also led to substantial accumulation of transferrin, a prerequisite for any CATL inhibitor in order to be trypanocidal.

Although we have recently shown that LU-102 at 10 μM did not significantly inhibit the trypsin-like activity of the trypanosomal proteasome in cell extracts [8], there might be the possibility that at the higher concentration of 25 μM the compound inhibits the proteasomal trypsin-like activity more readily facilitated by the intracellular reducing environment. To exclude this possibility, bloodstream forms of *T. brucei* were incubated with 25 μM LU-102 for 2 h and the proteasomal trypsin-like activity in cell extracts was subsequently measured using the fluorogenic trypsin-like peptide substrate Boc-LSTR-AMC. Under the experimental conditions, LU-102 inhibited the trypsin-like activity by 13.5% (Fig. 3F). However, the trypsin-like activity in trypanosomes treated with LU-102 was not statistically significantly different from that of control parasites (Fig. 3F). The observed extent of inhibition of the trypsin-like activity by LU-102 within trypanosomes is in line with that recently reported in trypanosome cell extracts (13.5% inhibition at 25 μM within cells compared to 8.7% inhibition at 10 μM in cell lysate) [8]. The finding shows that LU-102 also does not substantially inhibit the trypsin-like activity within trypanosomes. In addition, the observed limited inhibition of the proteasomal trypsin-like activity is certainly not sufficient to explain the trypanocidal activity of LU-102.

A previous chemical validation study using a variety of cysteine protease inhibitors provided evidence suggesting that *Tb*CATL rather than *Tb*CATB is essential to the survival of *T. brucei* bloodstream forms [6]. This conclusion was reached based on the observation that the CATB-specific inhibitor CA-074 displayed no trypanocidal activity although the compound inhibited almost completely the activity of *Tb*CATB within trypanosomes [6]. As all other inhibitors employed suppressed considerably the activity of both *Tb*CATL and *Tb*CATB, it remained unclear whether not both proteases needed to be inhibited in order to kill the parasite. In this study, we have now shown that inhibition of *Tb*CATL alone is sufficient to kill bloodstream forms of *T. brucei*. This was only possible as the vinyl sulfone compound LU-102 selectively inhibited the activity of *Tb*CATL in trypanosomes without affecting *Tb*CATB and the trypsin-like activity of the trypanosomal proteasome. The finding that *Tb*CATL is essential to the survival of bloodstream forms of *T. brucei* suggests that future drug development programmes should focus on the rational design of *Tb*CATL inhibitors.

References

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Figure legends

Fig. 1. Chemical structure of LU-102. The PubChem Compound Identifier (CID) of LU-102 is 71562351.

Fig. 2. (A) Effect of LU-102 on cysteine protease activity in cell extracts of *T. brucei*. Bloodstream forms of *T. brucei* 427-221a were harvested, washed once with PBS/1% glucose and lysed in 100 mM citrate, pH 5.0, 2% CHAPS on ice for 10 min. After centrifugation at 16873*g* for 5 min, aliquots of clarified cell extracts were treated with different concentrations of LU-102 (10-fold serial dilutions from 100 µM to 1 nM) in the presence of 10% DMSO for 30 min at room temperature. Controls were treated with 10% DMSO alone. Then, 16 µl of samples containing 1×10^6 and 1×10^7 cell equivalents for determining *Tb*CATB/L and *Tb*CATB activity, respectively, were added to 1984 µl measuring buffer (100 mM citrate, pH 5.0, 2 mM DTT) containing 5 µM Z-FR-AMC and Z-RR-AMC, respectively. After 30 min (*Tb*CATB/L) or 120 min (*Tb*CATB), the fluorescence of released AMC was measured at excitation and emission wavelengths of 360 nm and 460 nm in a BIORAD VersaFluor fluorometer. Open circles, *Tb*CATB/L activity; open squares, *Tb*CATB activity. Data are mean values ± SD of three experiments. (B) Trypanocidal activity of LU-102. Bloodstream forms of *T. brucei* 427-221a were seeded in 96-well plates in a final volume of 200 µl Baltz medium containing 2-fold serial dilutions of LU-102 (100 µM to 0.78125 µM) and 1% DMSO. Control cultures contained medium and 1% DMSO. The initial cell density was 1×10^4 trypanosomes/ml. After 24 h incubation at 37 °C in a humidified atmosphere containing 5% CO_2, 20 µl of a 0.5 mM resazurin solution prepared in sterile PBS was added and the cells were incubated for a further 48 h so that the total incubation time was 72 h. Thereafter, the plates were read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Data are mean values ± SD of three experiments.

Fig. 3. (A,B) Effect of LU-102 on Z-FR-AMC and Z-RR-AMC the hydrolysis activity within trypanosomes. Bloodstream forms of *T. brucei* 427-221a (2×10^7/ml) were incubated with 25

µM of LU-102 or 25 µM Z-FA-DMK in Baltz medium in the presence of 2.5% DMSO at 37 °C in a humidified atmosphere containing 5% CO_2. Control cultures were incubated in medium containing 2.5% DMSO. After 2 h incubation, cells were harvested by centrifugation and washed three times with PBS/1% glucose. Then, cell pellets (2×10^7/100 µl) were lysed in 100 mM citrate, pH 5.0, 2% CHAPS on ice for 10 min. Subsequently, lysates were centrifuged and clarified supernatants were used to determine *Tb*CATB/L and *Tb*CATB activity in measuring buffer (100 mM citrate, pH 5.0, 2 mM DTT) in the presence of 5 µM Z-FR-AMC and Z-RR-AMC, respectively. For *Tb*CATB/L activity (A), 10 µl of cell lysate corresponding to 2×10^6 cell equivalents were added to 1990 µl measuring buffer. For *Tb*CATB activity (B), 60 µl corresponding to 1.2×10^7 cell equivalents were added to 1940 µl measuring buffer. After 15 min (*Tb*CATB/L) and 60 min (*Tb*CATB) incubation at room temperature, respectively, the release of free AMC was measured at excitation and emission wavelengths of 360 and 460 nm, respectively, in a BIORAD VersaFluor fluorometer. Specific activities (pmol AMC released/min/cell) were calculated using a standard curve constructed with uncoupled AMC. Data are mean values ± SD of three experiments. (C,D) Effect of DTT on inhibition of cysteine peptidase activity in cell extracts of trypanosomes by LU-102. Cell extracts of bloodstream form *T. brucei* 427-221a (prepared as described in Fig. 2) were pre-treated with 25 µM LU-102 or the equivalent amount of DMSO (10%) alone in the absence or presence of 2.5 mM DTT for 30 min at room temperature. Then, 16 µl of samples containing $0.35\text{-}0.40 \times 10^6$ and $0.26\text{-}0.44 \times 10^7$ cell equivalents for determining *Tb*CATB/L and *Tb*CATB activity, respectively, were added to 1984 µl measuring buffer (see Fig. 2) containing 5 µM Z-FR-AMC and Z-RR-AMC, respectively. After 30 min (*Tb*CATB/L, C) or 120 min (*Tb*CATB, D), the fluorescence of released AMC was measured and specific activities calculated as described above. Data are mean values ± SD of three experiments. (E) Effect of LU-102 on the accumulation of fluorescein-labelled transferrin in trypanosomes. Bloodstream forms of *T. brucei* (1×10^7/ml) were incubated with 50 µg/ml fluorescein-labelled bovine transferrin in Baltz medium supplemented with 2% BSA in the presence of 25 µM LU-102 and 2.5% DMSO (orange line). Control cultures were treated with 2.5% DMSO alone (red line; negative control) or with 25 µM Z-FA-DMK plus 2.5% DMSO (blue line; positive control). After 2 h incubation, cells were washed twice with PBS/1% glucose and fixed with 2% formaldehyde/0.05% glutaraldehyde in PBS. Accumulated fluorescein-labelled transferrin within trypanosomes was determined using a CyFlow® Cube 6 flow cytometer. (F) Effect of LU-102 on the proteasomal trypsin-like activity within trypanosomes. Bloodstream forms of *T. brucei* 427-221a (2×10^7/ml) were incubated with 25 µM of LU-102 in Baltz medium in the presence of 2.5% DMSO at 37 °C in a humidified atmosphere containing 5% CO_2. Control cultures were incubated in medium containing 2.5% DMSO. After 2 h incubation, cells were harvested by centrifugation and washed three times with PBS/1% glucose. Then, cell pellets (1×10^7/100 µl) were lysed in 10 mM Tris, 0.1 mM EDTA, pH 7.0, 0.2% NP-40, 2 mM ATP and 1 mM DTT on ice for 10 min. Subsequently, lysates were centrifuged (16873*g* for 5 min) and clarified supernatants were used to determine the proteasomal trypsin-like activity in 50 mM HEPES, pH 7.5 with 5 µM Boc-LSTR-AMC. After 30 min, the fluorescence of released AMC was measured and specific activities calculated as described above. Data are mean values ± SD of three experiments.

Fig. 1

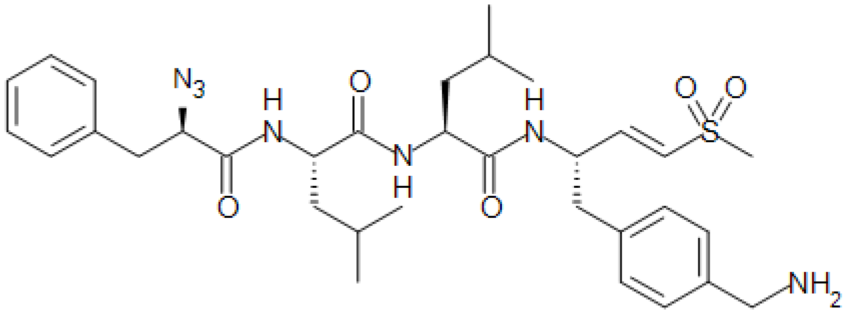
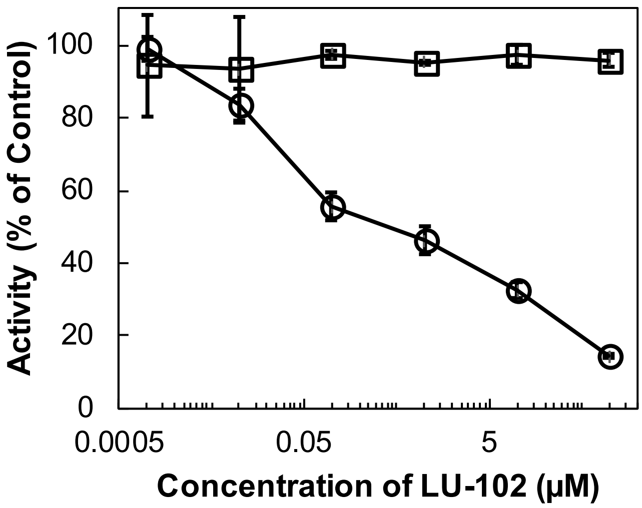


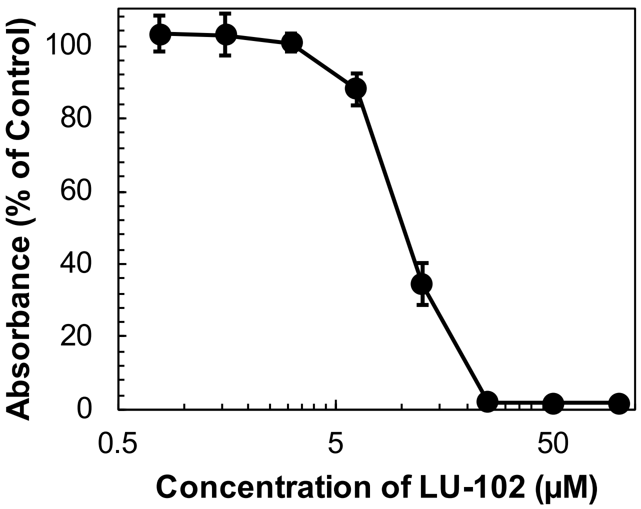
Fig. 2

A



Activity (% of Control)
0
20
40
60
80
100
0.0005
0.05
5
Concentration of LU-102 (μM)

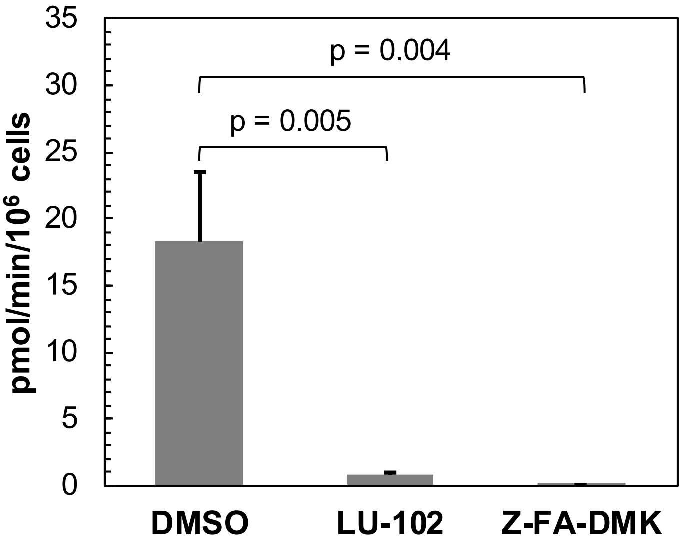
B



Absorbance (% of Control)
0
20
40
60
80
100
0.5
5
50
Concentration of LU-102 (μM)

Fig. 3

A


pmol/min/10^6 cells
35
30
25
20
15
10
5
0
p = 0.004
p = 0.005
DMSO
LU-102
Z-FA-DMK

B

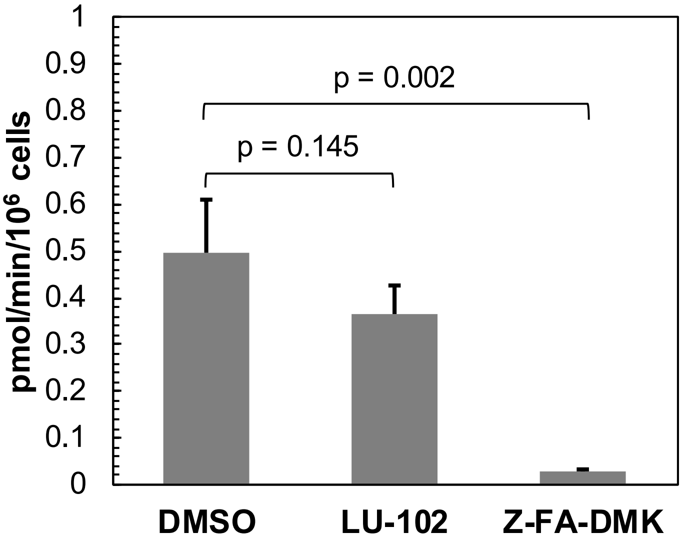
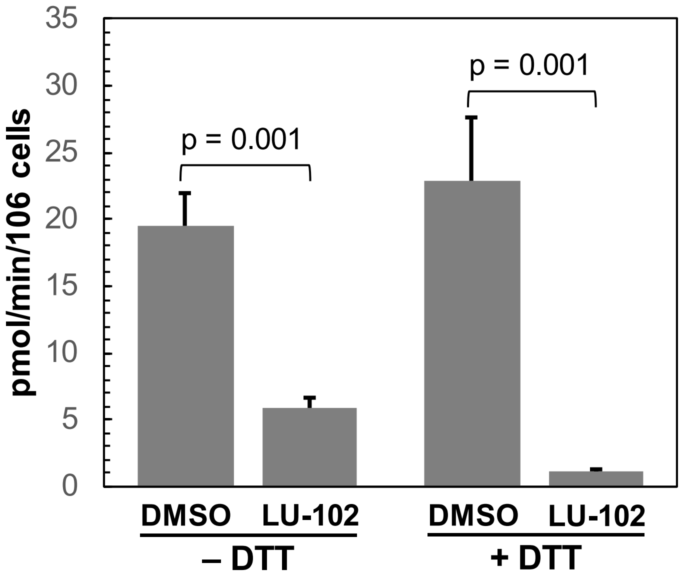

pmol/min/10^6 cells
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
p = 0.002
p = 0.145
DMSO
LU-102
Z-FA-DMK

Fig. 3 (continued)

C



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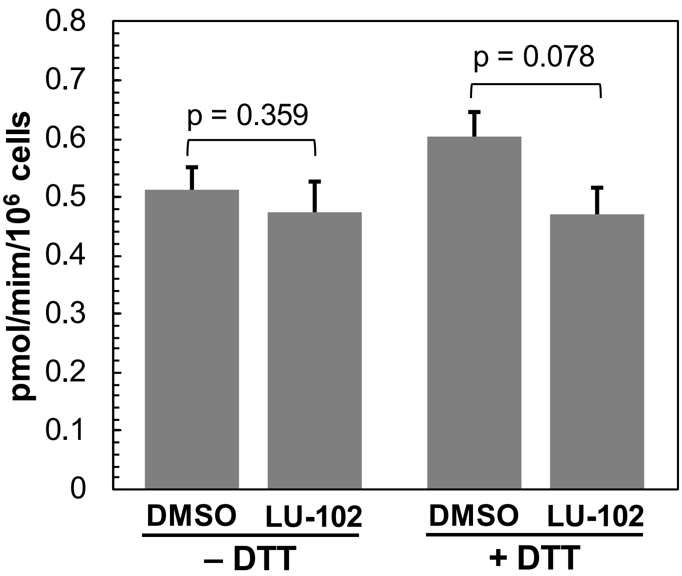
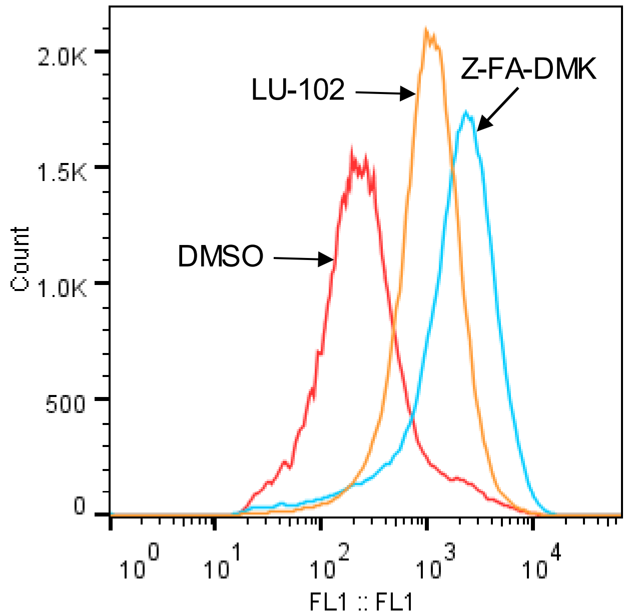


Fig. 3 (continued)

E



F