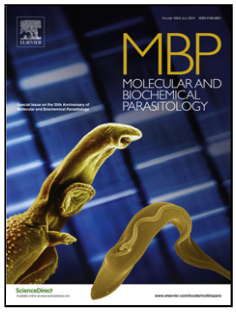

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Trypanosoma brucei: β2-selective proteasome inhibitors do not block the proteasomal trypsin-like activity but are trypanocidal

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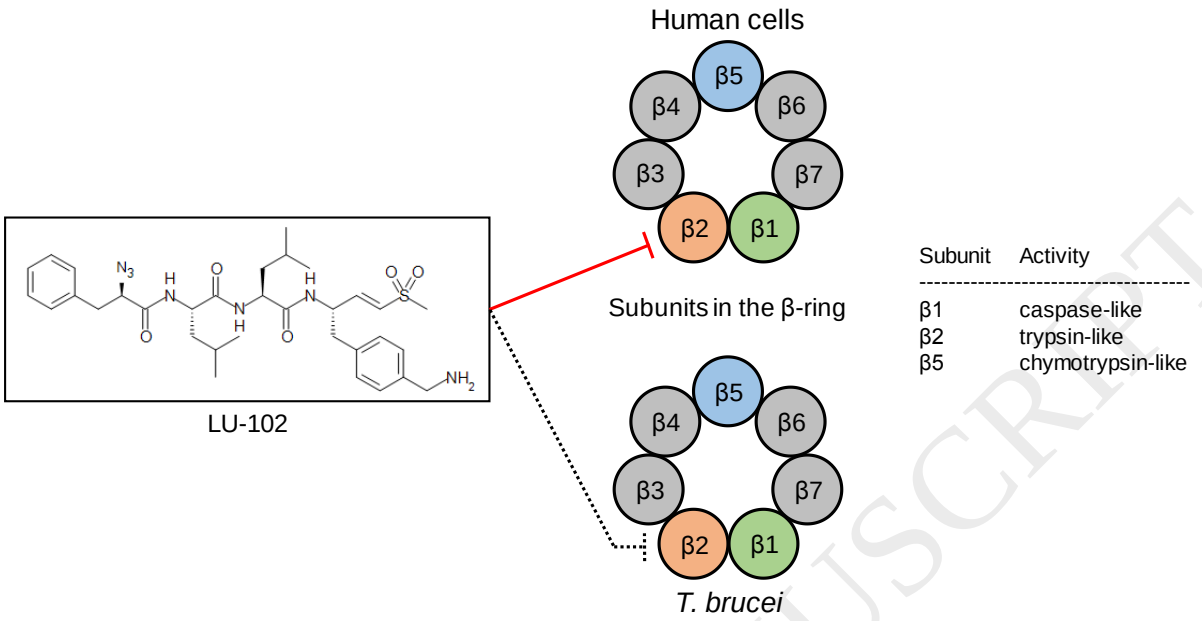
GRAPHICAL ABSTRACT



HIGHLIGHTS

- some β2-specific inhibitors display moderate trypanocidal activity.
- β2-specific inhibitors do not block proteasomal trypsin-like activity of *T. brucei*.
- study proves inhibitor sensitivity differences between human/*T. brucei* proteasome.

ABSTRACT

Previous studies indicated that the proteasome of the protozoan parasite *Trypanosoma brucei* is particularly sensitive to inhibition of the trypsin-like activity. In this study, three newly developed β2 subunit-specific inhibitor (LU-102, LU-002c and LU-002i) were tested for their ability to block the trypsin-like activity of the trypanosomal proteasome. At 10 µM, none of the compounds affected the proteasomal trypsin-like activity in cell lysates of bloodstream forms of *T. brucei*. On the other hand, leupeptin, a well-established β2 inhibitor, supressed the proteasomal trypsin-like activity within trypanosome cell lysates with a 50% inhibitory

concentration of 2 μM demonstrating the inhibitability of the trypsin-like activity of the *T. brucei* proteasome under the experimental condition. Nevertheless, two compounds (LU-102 and LU-002i) displayed moderate trypanocidal activity with 50% growth inhibition values of 6.9 and 8.5 μM, respectively. In the case of LU-102, it was shown that the trypanocidal activity of the compound was due to inhibition of the major lysosomal cysteine protease *Tb*CATL. The main finding of this study indicate substantial inhibitor sensitivity differences between the trypsin-like sites of the human and trypanosomal proteasomes. Whether these differences can be exploited for the design of anti-trypanosomal drug therapies remains to be shown.

Keywords:

Trypanosoma brucei

African trypanosomiasis

Proteasome

β2 subunit-specific inhibitors

The proteasome is a multi-subunit protease complex that plays a crucial role in the regulation of protein degradation in cells. Like all eukaryotic proteasomes, the 20 S core particle of the proteasome of the protozoan parasite *Trypanosoma brucei* has a cylindrical structure and is made up of four rings [1,2]. All essential seven α- and β-subunits have been identified as part of the trypanosomal 20 S proteasome [2,3]. Affinity-labelling experiments confirmed that also for the *T. brucei* 20 S proteasome the caspase-like activity, the trypsin-like activity and the chymotrypsin-like activity are associated with the β1, β2 and β5 subunits, respectively [2,4]. By using RNA interference to block selectively the expression of β-subunits, it was shown that the three catalytic subunits β1, β2, and β5 are vital for *T. brucei* [2,5]. However, this type of experiment does not conclusively prove that the activity of any of the catalytic subunits are essential for the survival of the parasite as RNAi of β1, β2 or β5 most likely leads to the instability in the structural integrity of the proteasome. Nevertheless, studies with inhibitors have shown that blocking the proteasome activity kills bloodstream forms of *T. brucei* [6]. This is corroborated by the identification of a selective inhibitor (GNF6702) of the trypanosomatid proteasome with potent *in vivo* efficacy, curing mice infected with *T. brucei*, *T. cruzi* and *Leishmania donovani* [7]. This compound inhibits the activity of the trypanosomtid proteasome through a non-competitive mechanism by binding at the interface between the non-catalytic β4 and catalytic β5 subunits, thereby blocking the chymotrypsin-like activity [7].

While there is structural and functional similarity between the trypanosomal and mammalian proteasome, there are substantial differences with respect to peptidase activity and inhibitor sensitivity. The trypanosomal proteasome has a high trypsin-like activity and a low chymotrypsin-like activity [1,2]. This is in contrast to the mammalian proteasome, which has a low trypsin-like activity and a high chymotrypsin-like activity. The difference in activity between trypanosomal and mammalian proteasomes is corroborated by their sensitivity towards inhibitors. Inhibitor studies have shown that the trypanosomal proteasome is particularly sensitive to inhibition of the trypsin-like activity [2,8-10]. The significant differences in peptidase activity and inhibitor sensitivity between the trypanosomal and mammalian proteasome make this enzyme complex a promising target for the development of urgently needed drugs for chemotherapy of African trypanosomiasis, a tropical neglected

disease affecting mainly poor communities in rural regions of sub-Saharan Africa [9]. Inhibitors targeting the proteasomal trypsin-like activity should display low cytotoxicity, as the host cells, in contrast to trypanosomes, rely much less on the trypsin-like activity of the proteasome.

So far, most proteasome inhibitors have been designed to target the chymotrypsin-like activity as they were developed as potential anti-cancer agents. However, previous work has shown that inhibitors of the trypsin-like activity sensitise myeloma cells to the antimyeloma drugs bortezomib and carfilzomib [12]. This led to the development of the improved trypsin-like site inhibitors LU-102, LU-002c and LU-002i (Fig. 1A) [13,14]. Whereas LU-102 is an inhibitor of the trypsin-like site of both constitutive (β2c) and immuno (β2i) 20S proteasome, LU-002c and LU-002i are β2c- and β2i-specific inhibitors, respectively [14].

In this study, we investigated whether the β2-specific inhibitors LU-102, LU-002c and LU-002i were able to inhibit the trypsin-like activity of the proteasome of *T. brucei*. We also evaluated the *in vitro* trypanocidal activity of the three compounds with bloodstream form trypanosomes.

The evaluation of the inhibitory activity of LU inhibitors was performed with extracts from bloodstream forms of *T. brucei* and human myeloid leukaemia HL-60 cells using specific substrates for the trypsin-like and chymotrypsin-like activity of the proteasome (Fig. 1B-E). At 10 μM, none of the inhibitors affected the activity of the trypsin-like activity of the trypanosomal proteasome (Fig. 1B). In the case of LU-002i, this was expected, as this compound is a specific inhibitor of the trypsin-like activity of the immunoproteasome [14] that is only expressed in immune cells. In contrast to the LU compounds, leupeptin, an established β2 inhibitor [15], blocked the trypsin-like activity of the trypanosomal proteasome by 90% at 10 μM (Fig. 1B). This result shows that the assay employed is capable of detecting the inhibition of the proteasomal trypsin-like activity in *T. brucei* cell extracts. In addition, further analysis revealed that leupeptin inhibited the trypsin-like activity of the trypanosomal proteasome in a dose-dependent manner with a half-maximal inhibitory concentration (IC_{50}) of 2 μM (Fig. 1C). This IC_{50} value is in good agreement with a previously determined IC_{50} value of 1 μM for leupeptin for inhibiting the labelling of the β2 subunit of purified *T. brucei*

proteasome with ^{125}I-Try-Leu-Leu-vinylsulfone [4]. Furthermore, control experiments with cell extracts from human HL-60 cell revealed that LU-102 and LU-002c inhibited the trypsin-like activity of the mammalian proteasome with similar apparent IC_{50} values of 0.27 and 0.21 μM, respectively (Fig. 1D). These IC_{50} values were within the range of IC_{50} values previously determined for LU-102 and LU-002c for inhibiting the constitutive proteasomal trypsin-like activity of Raji, HEK-293 and RPMI-8226 cells (0.007-1.8 μM) [14]. On the other hand, LU-002i did not inhibit the proteasomal trypsin-like activity in HL-60 cell extracts (Fig. 1D). The results obtained with HL-60 cell extracts confirm that under the experimental conditions LU inhibitors performed as expected with the mammalian proteasome. This observation indicates that the inability of the LU compounds to inhibit the trypsin-like activity of the trypanosomal proteasome is not due to an artefact. To exclude the unlikely possibility that the LU inhibitors inactivate the β5 subunit of the trypanosomal proteasome, the effect of the compounds on the chymotrypsin-like activity in *T. brucei* cell lysates was also studied. At 10 μM, none of the compounds inhibited the chymotrypsin-like activity of the trypanosomal proteasome (Fig. 1E). In contrast, bortezomib, a proteasome inhibitor primarily acting on the β5 subunit [16], blocked the chymotrypsin-like activity of the trypanosomal proteasome by 75% (Fig. 1E). These results show that the LU compounds are not inhibitors of the β5 subunit of the proteasome of *T. brucei*.

Despite being ineffective in inhibiting the trypsin-like activity of the trypanosomal proteasome, LU-102 and LU-002i showed moderate trypanocidal activity with 50% growth inhibition (GI_{50}) values of 6.9 and 8.5 μM, respectively (Fig. 2A). Interestingly, LU-002c, which, compared to LU-102, has a methyl group in the P3 position instead of an isobutyl group, did not display trypanocidal activity (Fig. 2A). Of the three compounds, only LU-102 exhibited cytotoxic activity with a GI_{50} of 8.2 μM against HL-60 cells (Fig. 2B). Similar cytotoxicities were previously reported for LU-102 against different myeloma cell lines [17]. The cytotoxic activity of LU-102 is most likely due to its ability to inhibit also cathespins [13] as inhibition of the proteasomal trypsin-like activity is not toxic to myeloma cells [12]. Therefore, we investigated whether the trypanocidal activity of LU-102 and LU-002i was the result of inhibition of the cathepsin L-like protease *Tb*CATL in trypanosomes. *Tb*CATL is the predominant lysosomal cysteine protease in bloodstream forms of *T. brucei* and essential to the

survival of the parasite [18]. Incubation of trypanosomes with 10 µM LU-102 for 2 h resulted in the inhibition of *Tb*CATL activity by 93.5% (Fig. 2C), which was sufficient to explain the growth inhibitory effect of the compound. However, LU-102 was not as powerful in inhibiting the *Tb*CATL activity as the established cathepsin L inhibitor Z-Phe-Ala-diazomethylketone (Fig. 2C) [19]. In contrast, treatment of trypanosomes with 10 µM LU-002c or LU-002i for 2 h did not affect the activity of *Tb*CATL (Fig. 2C). These findings indicate that the moderate trypanocidal activity of LU-102 is due to inhibition of *Tb*CATL while the target of LU-002i remains to be established.

Although LU-102 and LU-002c are potent inhibitors of the constitutive trypsin-like activity of the human proteasome, both compounds were ineffective in inhibiting the trypsin-like activity of *T. brucei*. This finding is fundamental as it clearly shows that human and trypanosome proteasome have different inhibitor sensitivities for their trypsin-like activity. It should be possible to exploit this difference to design agents selectively inhibiting the trypsin-like activity of the trypanosomal proteasome that could be developed into drugs for the treatment of African trypanosomiasis.

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Figure legends

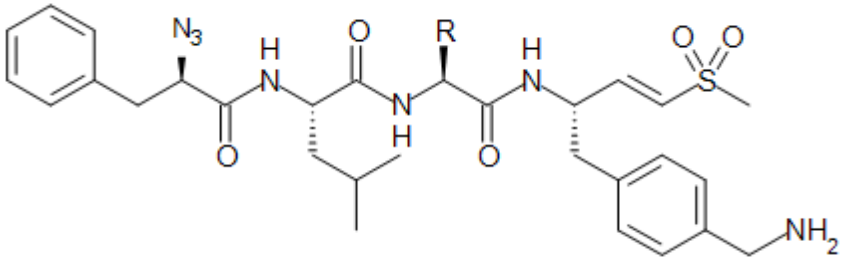
Fig. 1. (A) Chemical structure of LU inhibitors. (B-E) Effect of LU inhibitors on the activity of the proteasome of *T. brucei* and HL-60 cells. Bloodstream form trypanosomes or human myeloid HL-60 cells were harvested, washed with PBS/1% glucose and lysed in 10 mM Tris, 0.1 mM EDTA, pH 7.0, 0.2% NP-40, 2 mM ATP and 1 mM DTT on ice for 30 min. After centrifugation at 9800*g*, aliquots of clarified cell extracts were treated with inhibitors dissolved in 100% DMSO for 30 min at room temperature. Controls were treated with DMSO alone and the final concentration of DMSO was in all sample 1%. Trypsin-like activity and chymotrypsin-like activity of the treated cell extract samples were assayed in 50 mM HEPES, pH 7.5 with 5 µM Boc-Leu-Ser-Thr-Arg-AMC and 5 µM Suc-Leu-Leu-Val-Tyr-AMC, respectively. Release of free AMC was measured at excitation and emission wavelength of 360 nm and 460 nm, respectively, using a BIORAD VersaFluor fluorometer. Specific activities (pmol AMC released/min/cell) were calculated using a standard curve constructed with uncoupled AMC. (B) Effect of LU-102, LU-002c, LU-002i and leupeptin (LEU) on the trypsin-like activity of the proteasome of *T. brucei*. Trypanosome cell extracts were treated with 10 µM inhibitors or 1% DMSO and then assayed for proteasomal trypsin-like activity. Data are mean values ± SD of three experiments. The trypsin-like activity in cell extracts treated with LU-102, LU-002c and LU-002i was not statistically significantly different from the DMSO control ($p = 0.471$, 0.727 and 0.963, respectively). The trypsin-like activity in cell extracts treated with leupeptin (LEU) was statistically significantly different from the DMSO control ($p = 0.001$). (C) Dose-dependent inhibition of the trypsin-like activity of the proteasome of *T. brucei* by leupeptin. Trypanosomes cell extracts were treated with varying concentrations of leupeptin (10-fold dilution from 100 µM to 10 nM), or 1% DMSO (control), and then assayed for proteasomal trypsin-like activity. Data are mean values ± SD of three experiments. (D) Dose-dependent inhibition of the trypsin-like activity of the proteasome of HL-60 cells by LU-102 (circles), LU-002c (squares) and LU-002i (triangle). HL-60 cell extracts were treated with varying concentration of LU-102 or LU-002c (10-fold dilution from 10 µM to 1 nM), with 10 µM LU-002i, or with 1% DMSO (control), and assayed for proteasomal trypsin-like activity.

For clarity, only mean values of three experiments are shown. The standard deviations ranged from 2.0 to 12.4 percentage points (average 5.4±3.8 percentage points). (E) Effect of LU-102, LU-002c, LU-002i and bortezomib (BZB) on the chymotrypsin-like activity of the proteasome of *T. brucei*. Trypanosome cell extracts were treated with 10 µM inhibitors or 1% DMSO and then assayed for proteasomal chymotrypsin-like activity. Data are mean values ± SD of three experiments. The chymotrypsin-like activity in cell extracts treated with LU-102, LU-002c and LU-002i was not statistically significantly different from the DMSO control (p = 0.891, 0.937 and 0.429, respectively). The chymotrypsin-like activity in cell extracts treated with bortezomib (BZB) was statistically significantly different from the DMSO control (p = 0.007).

Fig. 2. (A,B) Trypanocidal and cytotoxic activity of LU inhibitors. Bloodstream forms of *T. brucei* (A) and human myeloid HL-60 cells (B) were seeded in 96-well plates in a final volume of 200 µl Baltz medium containing 2-fold serial dilutions of LU inhibitors (10, 5, 2.5, 1.25 and 0.625 µM) and 1% DMSO. Control cultures contained medium and 1% DMSO. The initial cell density was 1×10^4/ml for trypanosomes and 5×10^4/ml human cells. 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. For clarity, only mean values of three experiments are shown. The standard deviations ranged from 1.0 to 13.3 percentage points (average 3.8±2.6 percentage points). Circles, LU-102; squares, LU-002c; triangles, LU-002i. (C) Effect of LU inhibitors on the activity of the cathepsin-L like cysteine protease *Tb*CATL within trypanosomes. Bloodstream forms of *T. brucei* (2×10^7/ml) were incubated with 10 µM of LU-102, LU-002c, LU-002i or Z-FADK (Z-Phe-Ala-diazomethylketone) in Baltz medium in the presence of 1% DMSO at 37 °C in a humidified atmosphere containing 5% CO_2. Control cultures were incubated in medium containing 1% 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 100 mM citrate, pH 5.0, 2% CHAPS on ice for 10 min. Subsequently, lysates were centrifuged and clarified supernatants were used

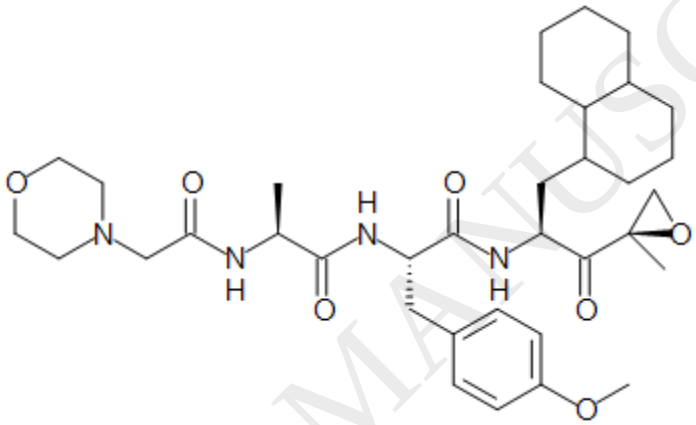
to determine *Tb*CATL activity in 100 mM citrate, pH 5.0, 2 mM DTT in the presence of 5 μM Z-FR-AMC (Z-Phe-Arg-7-amido-4-methyl coumarin) as fluorogenic substrate. After 30 min incubation at room temperature, the release of free AMC was measured at excitation and emission wavelengths of 360 and 460 nm, 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. The *Tb*CATL activity in cell extracts treated with LU-102 and Z-FADK was statistically significantly different from the DMSO control ($p = 0.001$ and 0.0008, respectively). The *Tb*CATL activity in cell extracts treated with LU-002c and LU-002i was statistically not significantly different from the DMSO control ($p = 0.863$ and 0.341).

Fig. 1A:



LU-102: R = $CH_2CH(CH_3)_2$

LU-002c: R = CH_3



LU-002i

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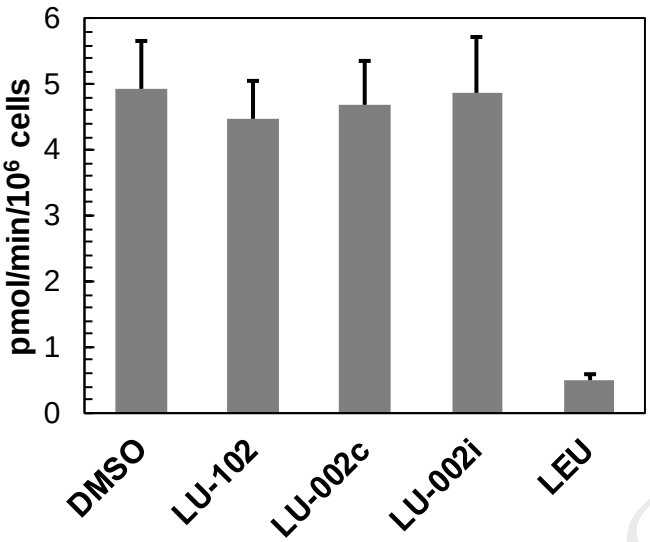

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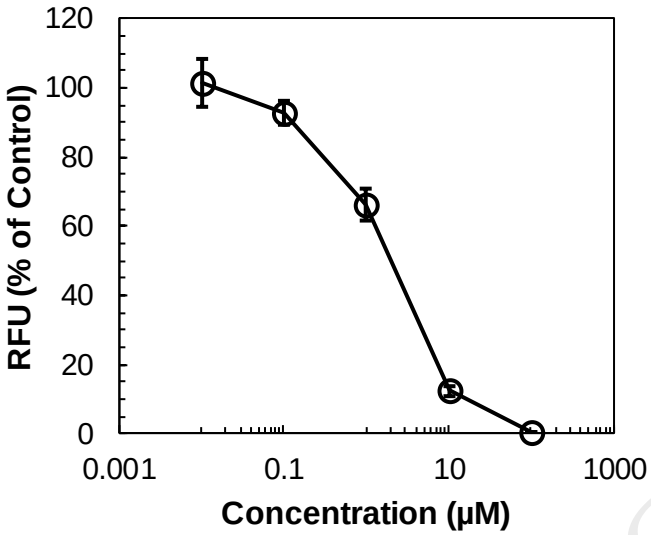

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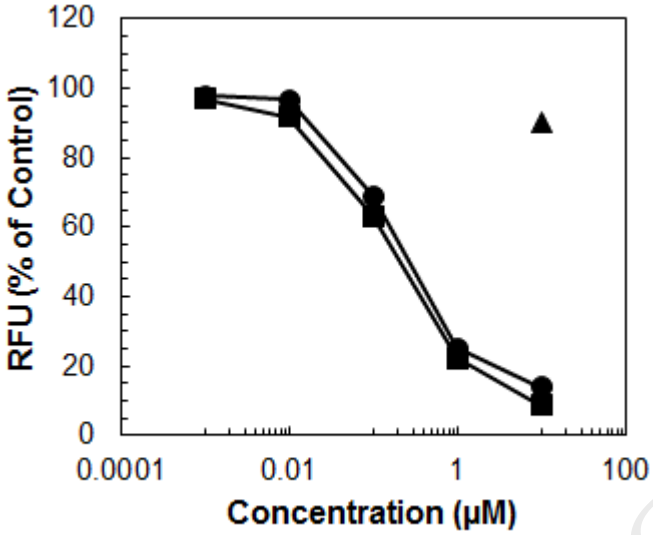

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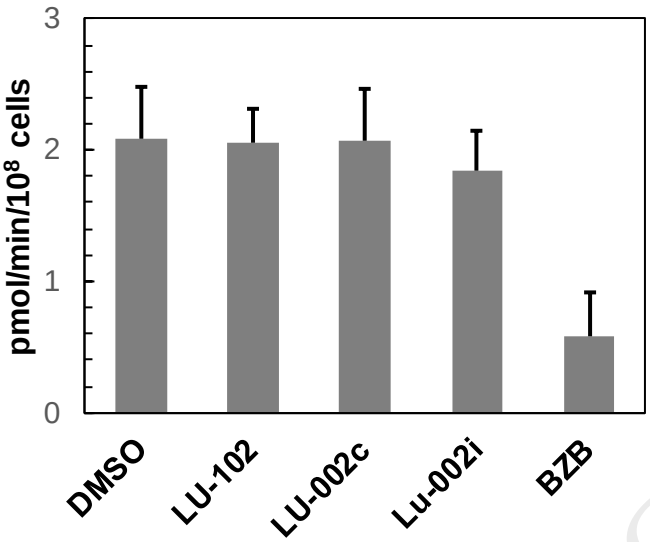

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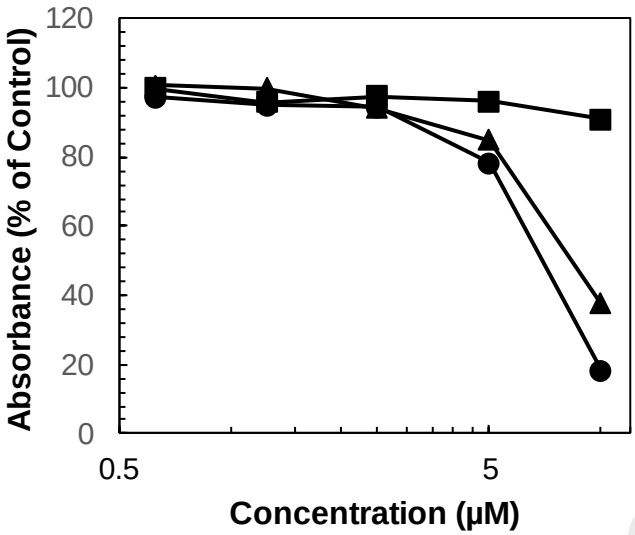

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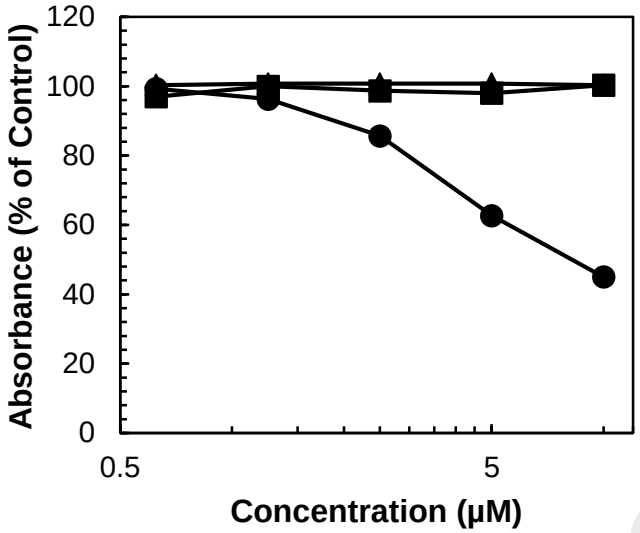

Fig. 2C: