Drug synergism: studies of combination of RK-52 and curcumin against rhodesain of *Trypanosoma brucei rhodesiense*

Roberta Ettari[1,], Santo Previti[1], Carla Di Chio,[1] Santina Maiorana, [1]Alessandro Allegra[2], Tanja Schirmeister[3] and Maria Zappalà[1]*

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Annunziata, 98168 Messina, Italy; [2]Division of Hematology, Department of General Surgery, Pathological Anatomy and Oncology, University of Messina, Via Consolare Valeria, 90100 Messina, Italy; [3]Institute of Pharmacy and Biochemistry, University of Mainz, Johann-Joachim-Becherweg 30, DE 55128 Mainz, Germany.

Supporting information

Biological assays

Rhodesa ininhibition assays

Preliminary screening with rhodesain was performed with inhibitor concentrations of 1000 µM or 100 µM to identify the range of activity of curcumin and **RK-52.** An equivalent amount of DMSO was used as negative control.

Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 10 µM) was determined continuously over a period of 30 min at room temperature, due to a time-dependent inhibition shown by both compounds. The assay buffer contains: 50 mM sodium acetate, pH = 5.5, 5 mM EDTA, 200 mM NaCl and 0.005 % Brij 35 to avoid aggregation and wrong-positive results. Enzyme buffer contains 5 mM DTT rather than Brij 35. Inhibitor solutions were prepared from stocks in DMSO. As first step, curcumin and **RK-52** were separately tested three times in duplicate in 96-well-plates in a total volume of 200 µL. Fluorescence of the product AMC of the substrate hydrolyses was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) at room temperature with a 380 nm excitation filter and a 460 nm emission filter. Results are expressed as IC_{50} values ± SD and have been calculated by fitting the progress curves to the 4-parameter IC_{50} equation by GRAFIT software 5.0 (GraFit, version 5.0.1.3; Erithacus Software Ltd.: London, 2006) :

$$y = \frac{y_{max} - y_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^{s}} + y_{min}$$

As second step, curcumin and RK-52 were tested in combination using 5 data points : 0.25 x $IC_{50F1+F2}$, 0.50 x $IC_{50F1+F2}$, $IC_{50F1+F2}$, 2 x $IC_{50F1+F2}$, 4 x IC_{50F+F2}.

Drug screening on *T. b. brucei* cultures

Antritrypanosomalactivites of RK-52 and curcumin were tested separately as well as in combination by using the ATPlite assay as described previously [1] with *T. brucei brucei* 449 cell line a descendant of the Lister strain 427.[2,3] Adenosine triphosphate (ATP) is a marker for cell viability, as cells die, their ATP content is depleted. The ATPlite assay based on the light emitting reaction of ATP and luciferin catalyzed by luciferase. Experiments were performed in a white 96-well plate (PerkinElmer) and each well contained 90 µL of HMI-9 medium with 2500 cells/mL. RK-52 (7.5 mM stock solution in DMSO), curcumin (12.5 mM stock solution in DMSO) or a combination thereof (RK-52:curcumin ratio 1:1.34, 5:6.7 mM) were diluted in multiple steps (first 1:3, then 1:10 and ten subsequent 1:2 dilutions in separated 96-well plates). 10 µL of the final 1:2 dilutions were added to the 90 µL cell suspension, leading to a final concentration range of 25.0 µM to 48.8 nM for RK-52, 41.7 µM to 81.4 µM for curcumin, 16.7 µM/22.3 µM to 32.6 nM/43.6 nM for combination of RK-52 and curcumin in the microplates. Addition of 0.3% of DMSO (corresponding to the highest DMSO concentration used in this assay) served as a negative control. As a positive control,to efficiently kill all cells, 10% of DMSO was added. Two plates with quadruplicates per compound/combination were incubated at 37 °C for 24 h. 50 µL of ATPlite 1step solution (PerkinElmer) was added to each well of the microplate and the bioluminescence measured with an Infinite® M200 PRO plate reader (Tecan Trading AG). A dose-response curve was obtained by plotting the measured bioluminescence values against the compound concentrations. The EC_{50} values were calculated by using GraFit version 5.013 (Erithacus Software Ltd.) and combined EC_{50} values were determined by addition of the concentrations of each compound.

References

(1) Crouch, S. P. M.; Kozlowski, R.;. Slater, K. J.; Fletcher, J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods*, **1993**, 160, 81-88

(2) Wagner, A.; Le, T. A.; Brennich, M.; Klein, P.; Bader, N.; Diehl, E.; Paszek, D.; Weickhmann, A. K.; Dirdjaja, N.; Krauth-Siegel R. L.; Engels, B.; Opatz, T.; Schindelin, H.; Hellmich, U. A. Inhibitor-Induced Dimerization of an Essential Oxidoreductase from African Trypanosomes. *Angew. Chem. Int*. Ed. **2019**, 58, 3640–3644

(3) Biebinger, S.; Wirtz, L. E.; Lorenz, P; Clayton, C. Vectors for inducible expression of toxic gene products in bloodstream and procyclic *Trypanosoma brucei. Molecular and Biochemical Parasitology.* **1997**, 85, 99 – 112