SHORT COMMUNICATION

Drug combination studies of PS-1 and quercetin against rhodesain of Trypanosoma brucei rhodesiense

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ABSTRACT
Rhodesain is a cysteine protease crucial for the survival of Trypanosoma brucei rhodesiense, the parasite able to induce the acute lethal form of Human African Trypanosomiasis. PS-1 is a synthetic peptidyl inhibitor of rhodesain, characterised by a picomolar binding affinity ($K_i = 1.1 \text{ pM}$). Thus, considering the well-known antiparasitic properties of quercetin, in this study, we decided to carry out drug combination studies of PS-1 and quercetin against rhodesain, according to Chou and Talalay method, which allowed us to obtain for the most relevant $f_a$ values a nearly additive effect for the reduction of rhodesain activity from 40% to 90%, thus considering a promising strategy their use in combination.

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1. Introduction
Human African Trypanosomiasis (HAT), caused by protozoa of Trypanosoma genus, is a parasitic disease occurring in more than 30 countries of sub-Saharan Africa, being an important cause of death, despite in the last years a consistent decrease in the number of new cases has been reported (Human African Trypanosomiasis 2017). In this...
context, rhodesain, the main cysteine protease of *T. brucei rhodesiense*, is considered a promising target for the drug-discovery process of the rhodesiense lethal form of HAT (Ettari et al. 2013; Ettari et al. 2016). As a matter of fact, rhodesain possesses several functions: (a) it is required by the trypanosome to cross the blood-brain barrier (BBB) (Nikolskaia et al. 2006), thus being responsible of the induction of the neurological stage; (b) it is involved in the evasion of host immune system by the degradation of host immunoglobulins and being involved in the turnover of variant surface glycoproteins (VSGs) of trypanosome coat (Barry and McCulloch 2001; Lalmanach et al. 2002); (c) it shows a relevant proteolytic activity in the lysosomes, being involved in the degradation of parasite proteins and intracellularly transported host proteins. For all these reasons rhodesain is currently considered a relevant target for HAT treatment (Ettari et al. 2013; Ettari et al. 2016).

In this therapeutic area, our research group has been actively involved in the last years in the development of novel rhodesain inhibitors for HAT treatment, leading recently to the identification of a potent rhodesain inhibitor, i.e., **PS-1** (Figure 1), endowed with a picomolar binding affinity ($K_i = 1.1$ pM) (Ettari, Previti, Maiorana, Amendola, et al. 2019).

In this study, considering the well-known antiparasitic activity of polyphenolic compounds, including quercetin (Mead and McNair 2006; Elizondo-Luévano et al. 2020; Ortiz et al. 2020; Faixova et al. 2021), and considering its variety of biological properties like the ability of quercetin to inhibit a panel of enzymes such as $\text{M}^{\text{pro}}$ and $\text{PL}^{\text{pro}}$ of SARS-Cov-2 (Derosa et al. 2021), its crucial role in the regulation and induction of apoptosis in cancer cells *in vitro* and *in vivo* (Davooodvandi et al. 2020), its anti-inflammatory activity in healthy volunteers (Boots et al. 2008), we decided to investigate its potential activity against rhodesain of *T. brucei rhodesiense*; thus, considering our previous studies on the opportunity to combine different types of therapeutic agents (Ettari, Previti, Maiorana, Allegra, et al. 2019; Ettari et al. 2020), we now report drug combination studies of **PS-1** and quercetin, according to Chou and Talalay method (Chou and Talalay 1984; Chou 2006, 2010) for the evaluation of the potential synergistic or additive effects for rhodesain inhibition.

2. Results and discussion

2.1. Inhibitory effect of curcumin and genistein alone and in combination against rhodesain

**PS-1** was synthesised as previously reported by our group (Ettari, Previti, Maiorana, Amendola, et al. 2019), while quercetin was purchased from Sigma Aldrich.
Quercetin and PS-1 were tested against recombinant rhodesain by using Cbz-Phe-Arg-AMC as fluorogenic substrate. We initially carried out a screening at 100 μM, 1 μM, 0.1 μM and 0.01 μM to evaluate the range of activity of the two potential inhibitors.

Quercetin and PS-1 were then subjected to detailed assays with 7 different inhibitor concentrations ranging from those that minimally inhibited to those that fully inhibited the enzyme. The obtained IC50 values from dose response-curves (Figure S1) are: 29.91 ± 0.78 μM for quercetin and 0.0018 ± 0.0003 μM for PS-1. The different IC50 values could be explained on the basis of the inhibition mechanism since PS-1 is a Michael acceptor, able to strongly covalently trap the active site thiol function of rhodesain, while quercetin does not bear any warhead.

In order to determine if a synergistic, additive or antagonistic effect occurs for the two drugs-combination, we selected five data points as reported in Table S1. In the case of experiments carried out with the combined doses of quercetin and PS-1, we obtained an IC50 value of 14.07 ± 1.53 μM, as shown in Figure S1.

The Median Effect Equation states that \( f_a/f_u = (D/D_m)^m \), where D is the dose, \( f_a \) and \( f_u \) are the affected and the unaffected fractions, respectively, of rhodesain activity, by the dose D; \( D_m \) is the dose required to produce the median effect (i.e., IC50), and ‘m’ is the Hill-type coefficient, signifying the sigmoidicity of the dose-effect curve (Chou and Talalay 1984; Chou 2006, 2010).

We then plotted the dose-response curves (Figure S1) as log (\( f_a/f_u \)) with respect to log (D), to generate the Median Effect Plot (Figure S2). In conclusion, by comparing the IC50 values of quercetin, PS-1 and of the combination PS-1 + quercetin and their related ‘m’ values for each Median Effect Plot (Figure S2), we found for quercetin IC50 = 29.91 μM and \( m_1 = 1.1754 \), for PS-1 IC50 = 0.0018 μM and \( m_2 = 1.5916 \) and for PS-1 + quercetin in combination (1:16617) IC50 = 14.07 μM and \( m_1,2 = 1.2640 \).

2.2. Drug interaction effect

The multiple drug effect analysis of Chou and Talalay, which is based on the median-effect principle, was used to examine the nature of the interaction between quercetin and PS-1 (Chou and Talalay 1984; Chou 2006, 2010). Determination of the synergistic versus additive versus antagonistic inhibitory effects of the combined treatment of rhodesain with quercetin and PS-1 were assessed using the Combination Index (CI), which was calculated as: \( CI = ([D_1]/[D_{50,1}]) + ([D_2]/[D_{50,2}]) \), where: \( D_{50,1}, D_{50,2} \) =the concentrations of quercetin and PS-1 that induced 50% of reduction of rhodesain activity (i.e., IC50); \( D_1, D_2 \) =the concentrations of quercetin and PS-1 in combination able to induce 50% of reduction of rhodesain activity.

The CI index was calculated using the Grafit software (Figure S3). The analysis of the CI (Figure S3 and Table S2) was carried out according to Chou rules (Chou 2006) obtaining for \( f_a \) ranging from 0.10 to 0.30 a slight synergistic effect, while for \( f_a \) ranging from 0.40 to 0.90 a nearly additive effect. On the contrary, no antagonistic effect was observed for the combination of the two inhibitors, thus considering a promising strategy their use in combination.
3. Experimental

See supplementary material.

4. Conclusion

In conclusion, in the present study, considering the biological activities of PS-1 and quercetin against rhodesain, we investigated their inhibitory properties in combination. Once calculated the single IC₅₀ values, we designed drug combination studies at five selected combined doses: the analysis of the CI, accordingly to Chou rules (Chou 2006) (Figure S3 and Table S2), clearly showed that for fₐ values ranging from 0.10 to 0.30 a slight synergistic effect was observed against rhodesain, while for fₐ values ranging from 0.4 to 0.9 an additive effect was detected by combining the two inhibitors; thus, in our future studies we will further investigate the toxicity of quercetin and PS-1 and the overall reduction of toxicity of the combination with respect to the single inhibitors, which is usually the main reason for the use of a drug combination.

Disclosure statement

No potential conflict of interest was reported by the authors.

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