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Drug Synergism: Studies of Combination of RK-52 and Curcumin against Rhodesain of *Trypanosoma brucei rhodesiense*

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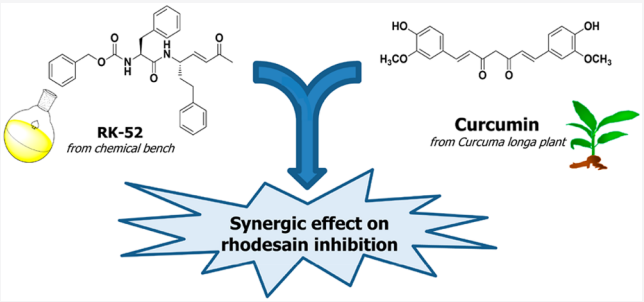
ABSTRACT: Rhodesain is an enzyme essential for the life of *Trypanosoma brucei rhodesiense,* a parasite causing a rapid-onset form of Human African Trypanosomiasis. **RK-52** is a synthetic inhibitor of rhodesain, characterized by an impressive k_{second} value (k_{second} = 67000 × 10^3 M^{-1} min^{-1}) and by a picomolar affinity toward the trypanosomal protease (K_i = 38 pM). Differently, curcumin, the golden multitarget nutraceutical obtained from *Curcuma longa* L., was proven to inhibit rhodesain non-competitively with an IC_{50} of 7.75 μM. In the present study, we carried out studies of a combination of RK-52 and curcumin toward rhodesain, by applying the Chou and Talalay approach, which led us to obtain a combination index <1 for the most relevant f_a values, which means a potent synergistic effect for the reduction of rhodesain activity from 40% to 99%.



KEYWORDS: *Rhodesain, Trypanosoma, drug combination studies, curcumin, synthetic inhibitor, synergism*

Human African Trypanosomiasis (HAT) is a parasitic disease occurring in sub-Saharan Africa, inducing mortality despite a significant decrease of novel cases recently having been observed.[1] HAT is induced by two subspecies of *Trypanosoma*: *T. brucei gambiense,* common in western and central Africa, able to cause a chronic HAT, and *T. brucei rhodesiense,* widespread in eastern and southern Africa, responsible for a rapid-onset high death rate HAT.[2] In the hemolymphatic stage of HAT, the parasite invades the bloodstream and then the lymph nodes and spleen inducing illness, muscle aches, and fatigue. If the parasite crosses the blood–brain barrier (BBB) a neurological stage occurs characterized by sleep disorders and death.[3] At present HAT therapy is based on only a few available drugs: pentamidine and suramin for the first-stage HAT, while the second-stage HAT is treated with eflornithine, melarsoprol, and nifurtimox. However, melarsoprol shows a high neurotoxicity, while eflornithine, whose use is safer, is active only against *T. b. gambiense*.[4] Thus, the first-line therapy for the treatment of the second-stage HAT is based on the coadministration of nifurtimox and eflornithine; more recently, in 2018, fexinidazole was introduced in therapy because of its activity against *T. brucei gambiense*.[5−7] Thus, new molecular targets have to be identified to develop innovative effective drugs for HAT therapy.

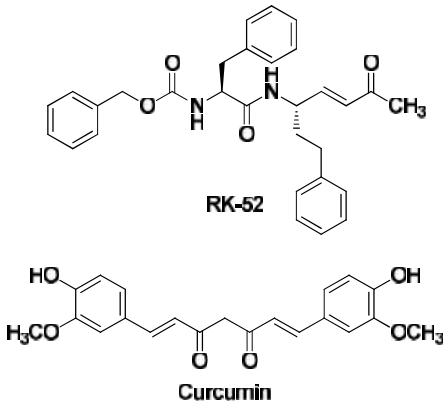


Figure 1. Structures of RK-52 and curcumin.

In this research area rhodesain, the key cysteine protease for the survival of *T. brucei rhodesiense,* is certainly a promising target to treat rhodesiense trypanosomiasis, the most lethal form of HAT.[8,9] Rhodesain is essential to permit the parasite to cross the BBB reaching the central nervous system, thus inducing the second-stage HAT; it takes part in the turnover of variant surface glycoproteins of the trypanosome coat[10] and in

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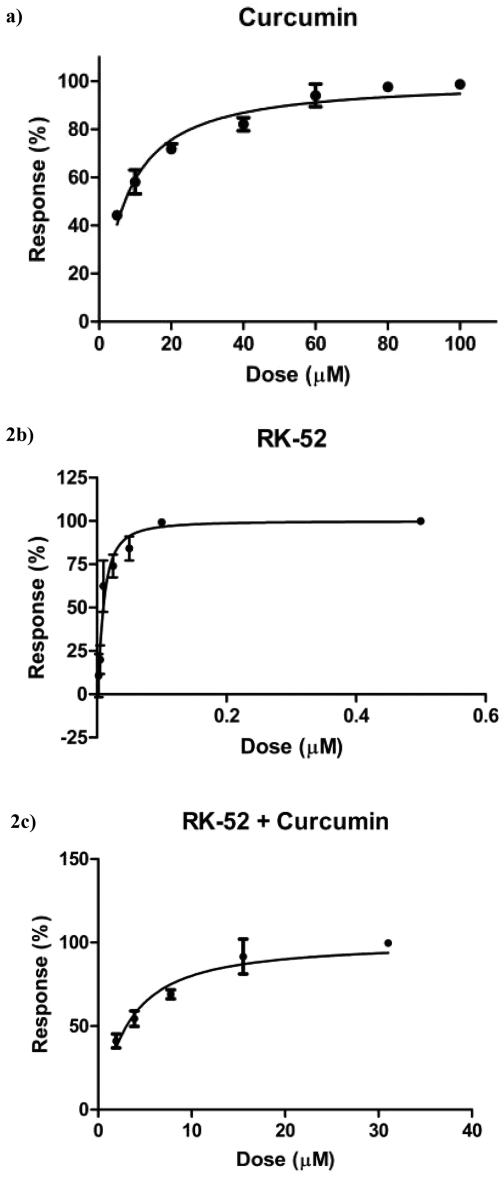

Figure 2. Dose response curves against rhodesain of curcumin[22] (a), **RK-52** (b), and **RK-52** + curcumin in combination (c). Each experiment was performed three times, each in duplicate.

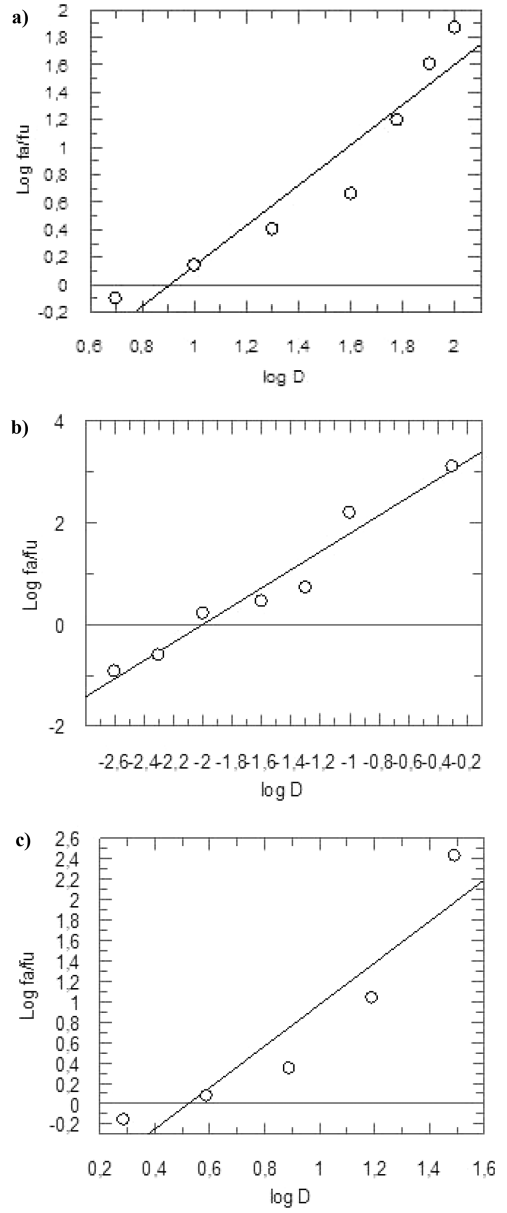

Figure 3. Median effect plot for curcumin[22] (a), **RK-52** (b), and the combination **RK-52** + curcumin (1:861) (c). f_a and f_u are the affected and the unaffected fraction, respectively, of rhodesain activity at the doses *D*.

evasion of the host immune system by degrading host immunoglobulins.[11] Moreover, rhodesain is predominant also in the lysosomes where it exerts its protease activity; thus, it is reasonable to consider the trypanosomal protease a promising target for HAT treatment.[12] In this context, in the past decade our research team has been working on the discovery of new rhodesain inhibitors as a novel strategy for HAT therapy,[13−20] leading to the identification of a potent irreversible rhodesain inhibitor, i.e. **RK-52** (k_{second} = 67000 × 10^3 M^{-1} min^{-1}) (Figure 1), endowed with a picomolar affinity toward trypanosomal protease (K_i = 38 pM).[21]

In the same field, we demonstrated also that curcumin (Figure 1), the golden multitarget nutraceutical obtained from *Curcuma longa* L., possesses an antitrypanosomal activity due to rhodesain inhibition (IC_{50} value of 7.75 μM).[22]

Table 1. Five Selected Doses for the Combination Experiments of Curcumin and RK-52

	Five selected doses (μM)				
	0.25 × IC_{50}	0.50 × IC_{50}	IC_{50}	2 × IC_{50}	4 × IC_{50}
Curcumin	1.93	3.87	7.75	15.5	31
RK-52	0.00225	0.0045	0.009	0.018	0.036
RK-52 + Curcumin	0.00225 + 1.93	0.0045 + 3.87	0.009 + 7.75	0.018 + 15.5	0.036 + 31

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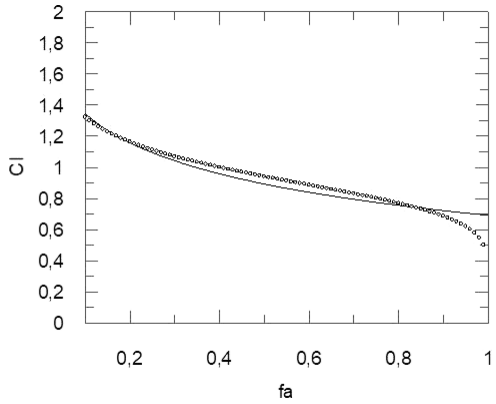

Figure 4. Combination index (CI) (*y*-axis) vs the fraction affected (*x*-axis), i.e. the reduction of rhodesain activity obtained by the combination **RK-52**−curcumin (1:861).

Table 2. Obtained Values of CI for the Combination RK-52−Curcumin (1:861) to Reduce Rhodesain Activity

Fraction affected (f_a)	Combination index (CI)	Diagnosis of combined effect
0.10	1,3227	Antagonism
0.20	1,1628	Additive
0.30	1,0694	Additive
0.40	0,9996	Synergism
0.50	0,9405	Synergism
0.60	0,8856	Synergism
0.70	0,8302	Synergism
0.80	0,7684	Synergism
0.90	0,6860	Synergism
0.99	0,4527	Synergism

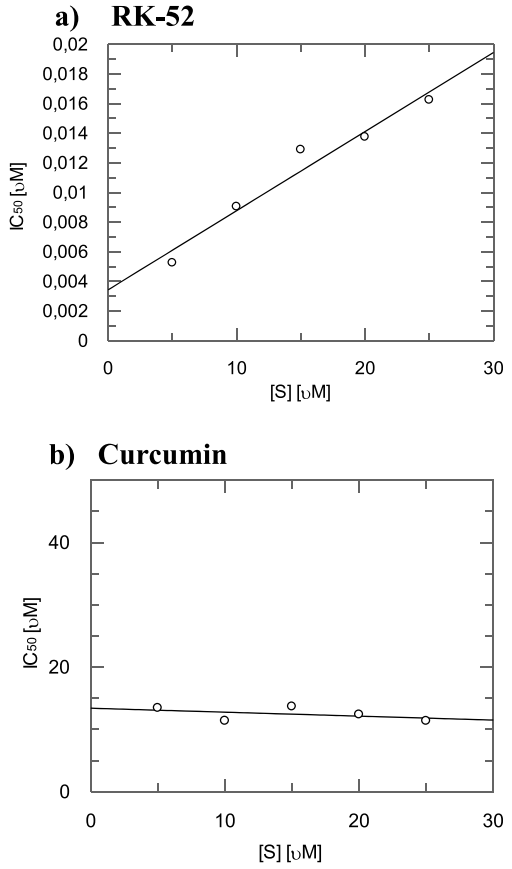

Figure 5. IC_{50} values of inhibition of rhodesain activity in dependence of the substrate concentration (5, 10, 15, 20, and 25 μM) for RK-52 (a) and curcumin (b).

In this study, we now discuss studies of a combination of **RK-52** and curcumin, by applying the Chou and Talalay approach,[23,24] to evaluate if additive or synergistic effects occur in rhodesain inhibition.

RK-52 was synthesized as previously reported by our group.[21] Curcumin was purchased from Sigma-Aldrich.

Rhodesain was obtained as previously described by Caffrey et al.[25] Curcumin and **RK-52** were then separately tested in three independent experiments, each performed in duplicate.

Both compounds were subjected to detailed assays at 7 concentrations, starting from the minimum dose required for the enzyme inhibition to that necessary to fully suppress rhodesain activity. More specifically, we used 0.5 μM, 0.1 μM, 0.05 μM, 0.025 μM, 0.010 μM, 0.005 μM, and 0.0025 μM for **RK-52**, while 100 μM, 80 μM, 60 μM, 40 μM, 20 μM, 10 μM, and 5 μM have been used for curcumin.

Biological evaluation against rhodesain was carried out using Cbz-Phe-Arg-AMC as fluorogenic substrate. IC_{50} values have been obtained from dose response-curves shown in Figure 2: 7.75 ± 1.53 μM for curcumin[22] and 0.009 ± 0.0008 μM for RK-52. Subsequently, five data points for both compounds (1/4 × IC_{50}, 1/2 × IC_{50}, IC_{50}, 2 × IC_{50}, and 4 × IC_{50}, Table 1) have been selected, with the aim to verify the possible synergistic, additive, or antagonist effect in the combination assay of the inhibitors. In this case, the combination of **RK-52** and curcumin provided an IC_{50} value of 3.05 ± 0.21 μM.

The median effect equation described by Chou states that $f_a/f_u = (D/D_m)^m$, where f_a and f_u represent the affected and the unaffected fractions, respectively, of the target enzyme, by the dose D; D_m corresponds to IC_{50}, able to produce the median effect, while m represents the Hill-type coefficient, which means the sigmoidal trend (or S-shaped) of the dose-reponse curve.[23,24]

The median effect plot was generated plotting the three dose−response curves as log (f_a/f_u), on the *y*-axis, with respect to log (D), on the *x*-axis (Figure 3). This allowed us to obtain the IC_{50} values of the three different evaluations, together with the related m coefficients. In detail, curcumin showed IC_{50} = 7.75 μM and m_1 = 1.4681, **RK-52** IC_{50} = 0.009 μM and m_2 = 1.7841 while for the combination assay we found IC_{50} = 3.05 μM and $m_{1,2}$ = 2.0305, with a molar ratio **RK-52**/curcumin of 1:861.

In order to determine the inhibitory effect given by curcumin and **RK-52**, the multiple drug effect evaluation reported in the literature by Chou and Talalay was used.[23,24] In this contest, the combination index (CI) provides the nature of the inhibition toward the target enzyme, when two drugs are evaluated simultaneously. In particular, three effects have been demonstrated for CI > 1, CI = 1, and CI < 1, which correspond to an antagonistic, additive, and synergistic impact, respectively.

The CI for mutually exclusive drugs was calculated as follows:

$$CI = [(D)_1/(D_{50})_1] + [(D)_2/(D_{50})_2] + [(D)_1(D)_2]/[(D_{50})_1(D_{50})_2]$$

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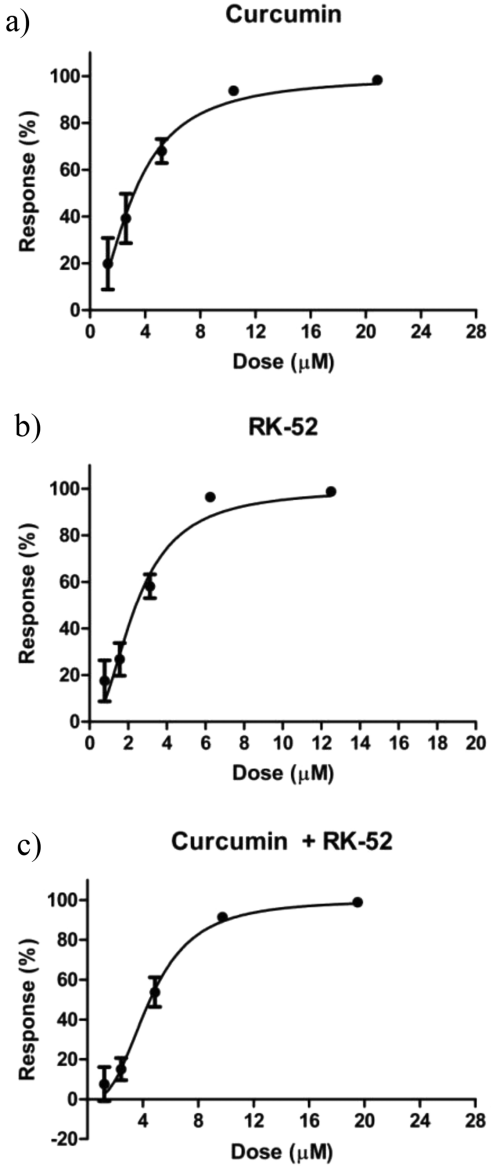

Figure 6. Dose response curves for the inhibition of *Trypanosoma brucei brucei* growth for curcumin (a), **RK-52** (b), and **RK-52** + curcumin in combination (c). Each experiment was performed three times, each in duplicate.

where $(D_{50})_1$ and $(D_{50})_2$ represent the IC_{50} values for curcumin and **RK-52** when they are evaluated alone, while $(D)_1$ and $(D)_2$ are the concentrations of both compounds able to produce 50% of rhodesain inhibition when they are used in combination.

Grafit software was used to determine the CI (Figure 4). The obtained values show a clear synergistic effect when the most significant f_a is ranging from 0.40 to 0.90, while for f_a = 0.10 and f_a ranging from 0.20 to 0.30, an antagonist and additive effect was recorded, respectively (Table 2).

Finally, since a synergism at all the relevant fa values was observed between the two rhodesain inhibitors we further investigated if the inhibition is competitive with respect to the substrate by determining the IC_{50} in dependence of five substrate concentration (5, 10, 15, 20, and 25 μM).

In the case of **RK-52** (Figure 5a) we found linearly increasing IC_{50} values with increasing substrate concentration proving that **RK-52** binds to the active site. On the contrary, in the case of curcumin (Figure 5b) with increasing substrate concentration no variation of IC_{50} was observed thus suggesting that curcumin binds to an allosteric site.

Curcumin, **RK-52**, and the combination **RK-52** + curcumin were also tested against *T. brucei brucei* (Figure 6). In these assays we obtained IC_{50} values of 3.12 ± 0.43 μM, 2.33 ± 0.29 μM, and 4.64 ± 0.35 μM for curcumin, **RK-52**, and **RK-52** + curcumin in combination (molar ratio 1:1.34), respectively.

The comparable activity of curcumin with respect to **RK-52** against *T. b. brucei,* with respect to the very different activity against rhodesain, could be explained considering that the antitrypanosomal activity of curcumin might be due, in addition to the inhibition of rhodesain, to the interaction with other proteases that can be found in the trypanosomal intracellular *environment.*

We have also to consider that the activity against *T. b. brucei* is also related to the capability of a drug to cross the cellular membranes, which is certainly more easy for low molecular weight molecules such as curcumin, with respect to **RK-52**.

All in all, in this study starting from the activities of **RK-52** and curcumin against rhodesain, we investigated the activity of their combination, obtaining for the most significant f_a values (from 0.40 to 0.90) a synergistic effect for rhodesain inhibition. The combination curcumin + **RK-52** showed an IC_{50} value against *T. b. brucei* of 4.64 μM; thus, it is reasonable in future studies to further investigate the overall reduction of toxicity of the combination with respect to the single inhibitors, which is normally the main reason for the use of drug combinations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00635.

Rhodesain inhibition assays; antitrypanosomal assays (PDF)

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All authors approved the content of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HAT, Human African Trypanosomiasis; BBB, blood−brain barrier; VSGs, variant surface glycoproteins; Cbz, carbobenzyloxy; Phe, phenylalanine; Arg, arginine; AMC, 7-amino-4-methyl-coumarin; f_a, fraction affected; f_u, fraction unaffected; CI, combination index

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