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In vitro antimalarial activity of hyperforin, a prenylated acylphloroglucinol. A structure–activity study

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Abstract—The antimalarial activity of hyperforin, a phenol-like compound that can be easily absorbed orally, and a series of derivatives variously modified on the cyclohexatrienone system was investigated. Hyperforin was active against *Plasmodium falciparum* with an IC_{50} value in the micromolar range, and the activity was not critically dependent on either its phenol-like sensitivity to autooxidation or the presence of unsaturation on the prenyl residues. Related phloroglucinols like the hop β-acids and the enantiomers of usnic acid showed only marginal activity, suggesting that hyperforin is a new antimalarial chemotype.

With over 500 million cases worldwide, and a mortality of 1.2 million people per year, the medical and social relevance of malaria can hardly be overestimated. Children and pregnant women are especially at risk, but, despite great efforts to develop a vaccine, chemotherapy is still the method of choice for the treatment of malaria. Because of its efficacy and low cost, chloroquine continues to play a major role for malaria treatment in primary health care. Unfortunately, resistant strains of *Plasmodium falciparum*, the most dangerous of the four species infecting humans, have emerged in the past decade, making the discovery of new antimalarial agents with a novel mode of action a global health priority. Genomic and biochemical studies on *Plasmodium* have identified a range of molecular targets for drug discovery, but the mechanism of action of the currently used antimalarial drugs is still not fully understood.

Plant natural products have played a key role in the discovery of antimalarial agents, as testified by quinine and artemisinin, and turning to natural products has been arguably the single most successful strategy for the discovery of new antimalarial agents. Within plant secondary metabolites, a host of phenolics and polyphenolics show antimalarial activity, presumably because of their capacity to interfere with mitochondrial electron transfer. Though potently active in vitro, these compounds generally show a narrow therapeutic window and little if any selectivity for the parasite, and have therefore been little investigated in terms of structure–activity relationships. On the other hand, there is a growing evidence that tannins can exert a range of specific activities, showing a bioactivity pattern that transcends their polyphenolic character.[1] Given the easy availability of many plant phenolics and phenolic-like agents, we have decided to further investigate this issue, using hyperforin compound (**1**) as a lead structure. Hyperforin is the major lipophilic constituent of St. John's wort (*Hypericum perforatum* L.) and, just like a polyphenolic compound, shows an oxygen reactive enol moiety. The oxygen sensitivity of hyperforin is somewhat dependent on the medium, being inversely related to polarity. While this very unusual behavior has not yet been fully elucidated, it is not unconceivable to assume that in polar solvents the hydrophobic collapse of the prenyl group shields the reactive enol moiety from the attack of oxygen.[2] While polyphenolics cannot be taken up in biological membranes because of their hydrophilic nature, hyperforin is fat-soluble,[3] and could easily penetrate membranes. This lipophilicity could translate into a specific trophism for mitochondria, a

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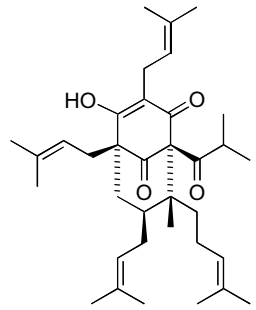
lipophilic milieu rich in activated oxygen species, where hyperforin might act as a biological analogue of quinones. Furthermore, hyperforin mimics the activity of monensin, a known antimalarial agent, on several monoamine neural transporters,[4] while a series of acylphloroglucinols related to hyperforin have been reported to show antiplasmodial activity in vitro and/or in vivo.[5,6] Taken together, these considerations provided a rationale for investigating the antimalarial activity of hyperforin and related analogues.

The analogues **2**–**12**[7–9] were prepared to covalently stabilize the enolized β-diketone moiety by O-acylation (**2**, **3**)[10], O-alkylation (**4, 6, 9**), α-alkylation (**5**), or oxidative α-functionalization (**7**, **8**, **9**, **10**).[8,11] The rearranged dienol lactone **11** was prepared, along with **7**–**9**, by hydrogen peroxide oxidation of the natural product.[9] Octahydrohyperforin (**12**),[12] readily prepared by hydrogenation of hyperforin, was included in the library to explore the role of the prenyl double bonds surrounding the reactive and enolized β-dicarbonyl moiety.



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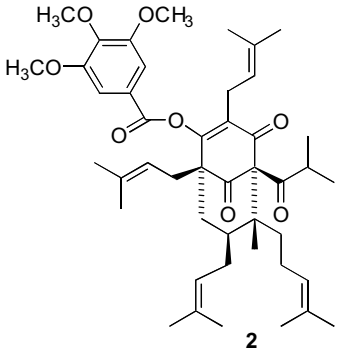

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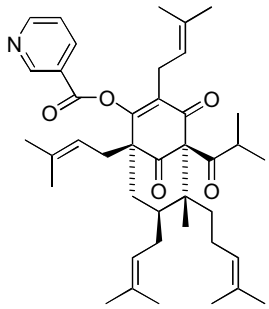

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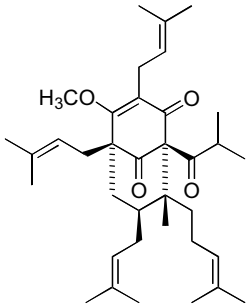

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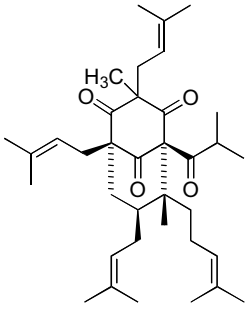

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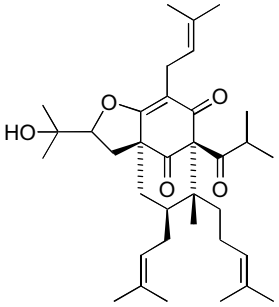

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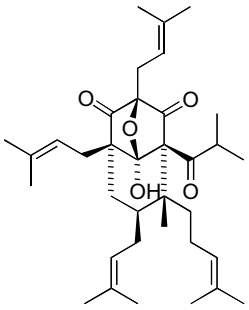

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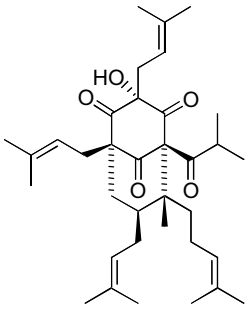

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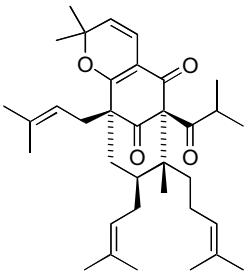

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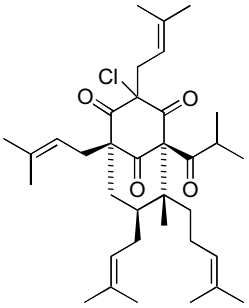

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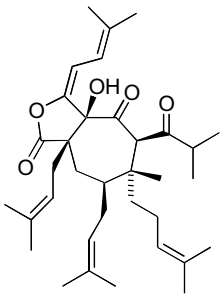

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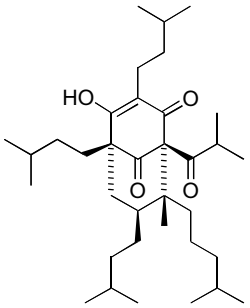

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Table 1. In vitro antiplasmodial activity of compounds **1**–**12**

	P. falc. K1		Cytotox. L6	
	IC-50	Ref drug	IC-50	Ref drug
Hyperforin (**1**) DCHA	1.5	0.062	2.21	0.017
Hyperforin (**1**) Li	2.1	0.062	3.29	0.017
Adhyperforin (**1a**) DCHA	1.4	0.062	2.85	0.017
Octahydrohyperforin (**12**) DCHA	1.4	0.062	3.12	0.017
Octahydrohyperforin (**12**) Li	2.7	0.062	2.12	0.017
Hyperforin trimethoxybenzoate (**2**)	>27	0.089	nt	
Hyperforin nicotinate (**3**)	4.8	0.089	23.9	0.017
7-OMe hyperforin (**4**)	7.8	0.089	nt	
8-Me hyperforin (**5**)	>27	0.089	nt	
Furohyperforin (**6**)	1.7	0.062	16.1	0.017
Hyperforin hemiacetal (**7**)	2.0	0.062	16.1	0.017
8α-hydroxyhyperforin (**8**)	0.6	0.062	19.7	0.017
Dehydrohyperforin (**9**)	8.6	0.089	nt	
8-Cl hyperforin (**10**)	6.7	0.089	nt	
(**11**)	3.8	0.089	36	0.017
Usnic acid (+)	15.3	0.089	nt	
Usnic acid (−)	16.1	0.089	nt	
Hop β-acids	7.4[a]	0.089	nt	

Data are expressed in micromolar concentration. The reference drug for *P. falciparum* was chloroquine and for the L6 cells it was podophyllotoxin. nt, not tested.

[a] This datum is in μg/mL because hop acids represent a mixture of homologues.

Hyperforin (**1**) was assayed as its stable lithium salt and showed only modest antimalarial activity (IC_{50} = 2.1 μM).[13] The corresponding dicyclohexylammonium salt showed a similar potency (1.5 μM), suggesting that the counter ion has only a marginal effect on the activity of the natural product. The homologue adhyperforin (**1a**) (assayed as dicyclohexylammonium salt) showed a similar activity (1.4 μM), indicating that the length of the biosynthetic acyl starter is not critical, at least for modest increases of length. Both hyperforin and adhyperforin were endowed with an intrinsic toxicity that, though modest, was nevertheless indicative of a narrow therapeutic index (see Table 1.)

O-Acylation of the enol moiety of hyperforin afforded compounds prone to enzymatic hydrolysis, and the marked difference of activity between the electron-poor and more hydrolysis-sensitive nicotinate **3** (IC_{50} = 4.8 μM) and the electron-rich and more hydrolytically stable trimethoxybenzoate **2** (IC_{50} > 27 μM) might simply be related to stability in the assay medium.

The enol ethers **4** and **9** show a decreased activity (7.8, 8.6 μM, respectively), while the stable analogue furohyperforin (**6**) showed a similar potency as the natural product (1.7 μM), suggesting a specific role for the oxygen-sensitive enolized β-dicarbonyl. This was further confirmed by the observation that the introduction of an oxygen or an halogen at the α-carbon of the β-dicarbonyl (compounds **7**, **8**, and **10**) substantially maintained or even increased the activity (6.7 μM for **10**, 2.0 μM for **7**; 0.6 μM for **8**). Conversely, α-alkylation as in **5** was detrimental for activity (>27 μM).

Hydrogenation of the prenyl appendages was irrelevant for activity (IC_{50} = 1.4 μM for the dicyclohexylammonium salt of **12** and 2.7 μM for its corresponding lithium salt). Remarkably, skeletal rearrangement was tolerated, as shown by the low micromolar IC_{50} of **11** (3.68 μM).

With this database of antimalarial activity at disposal, it was interesting to assess how the chemical modifications investigated translated in terms of cytotoxicity in L6 cells. Hyperforin, adhyperforin, and octahydrohyperforin demonstrate a certain cytotoxicity, and an overall limited therapeutic index. On the other hand, some analogues modified at the β-dicarbonyl system showed a marked decrease of cytotoxicity, with the selectivity index climbing from around 1 to 10–33 for the three more potent antimalarial agents (**6, 7, 8**).

Taken together, these observations show that (a) hyperforin, a phenol-like compound that can be easily absorbed orally,[14] can inhibit *Plasmodium* growth at micromolar concentrations; (b) the activity is not critically dependent on its phenol-like sensitivity to autooxidation, and (c) that the prenyl double bonds are not necessary for the activity. Since other phloroglucinols like the mixture of hop β-acids and the enantiomers of usnic acid showed only marginal activity (IC_{50} 7.4 μg/mL and 15.3 or 16.1 μM, respectively) (Table 1), hyperforin qualifies as a new antimalarial chemotype.

Acknowledgment

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7. Hyperforin dicyclohexylammonium or lithium salts were supplied by INDENA SpA. Hyperforin 1,8 hemiacetal (**7**), furohyperforin (**6**), dehydrohyperforin (**9**), 8-α-hydroxy hyperforin (**8**), 7-OMe hyperforin (**4**), 8-Me hyperforin (**5**) were prepared from hyperforin dicyclohexylammonium salt as reported in Ref. 8; compound (**11**) was prepared by H_2O_2 oxidation of hyperforin as reported in Ref. 9.
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10. Hyperforin trimethoxybenzoate (**2**). White crystals (MeOH), mp 82 °C; $[\alpha]_D^{20}$ +34.7 ($CHCl_3$, c 1); ^{1}H NMR (300 MHz, $CDCl_3$): 1.95 (m, H-4), 2.12 (dd, 13.5, 3.7, H-5), 1.43 (m, H-5′), 2.02 (m, H-11), 1.15 (d, 6.6, H-12), 1.04 (d, 6.6, H-13), 1.06 (s, H-14), 1.93 (m, H-15), 1.49 (m, H-15′), 2.15 (H-16), 1.93 (m, H-16′), 5.09 (m, H-17), 1.68 (s, H-19), 1.63 (s, H-20), 2.19 (m, H-21), 1.82 (m, H-21′), 5.11 (m, H-22), 1.75 (s, H-24), 1.63 (m, H-25), 3.14 (dd, 14.9, 7.1, H-26), 2.92 (dd, 14.1, 7.0, H-26′), 5.09 (m, H-27), 1.59 (s, H-29), 1.57 (s, H-30), 2.61 (dd, 15.3, 6.5, H-31), 2.34 (dd, 15.3, 6.0, H-31′), 5.09 (m, H-32), 1.57 (s, H-34), 1.59 (s, H-35), 7.34 (s, Ar 2+6), 3.98 (s, OCH_3), 2× 3.93 (s, OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$): 206.5 (s, C-1), 84.7 (s, C-2), 50.2 (s, C-3), 42.5 (d, C-4), 38.5 (t, C-5), 57.7 (s, C-6), 163.2 (s, C-7), 134.7 (s, C-8), 193.7 (s, C-9), 208.6 (s, C-10), 43.2 (d, C-11), 20.6 (q, C-12), 21.4 (q, C-13), 13.9 (q, C-14), 36.7 (t, C-15), 25.2 (t, C-16), 125.0 (d, C-17), 131.6 (s, C-18), 25.9 (q, C-19), 17.9 (q, C-20), 27.3 (t, C-21), 123.1 (d, C-22), 133.0 (s, C-23), 26.0 (q, C-24), 17.9 (q, C-25), 24.2 (t, C-26), 120.2 (d, C-27), 133.9 (s, C-28), 25.8 (q, C-29), 18.2 (q, C-30), 30.5 (t, C-31), 119.7 (d, C-32), 134.1 (s, C-33), 25.8 (q, C-34), 18.4 (q, C-35, 107.9 (d, Ar 2+6), 61.3 (s, OCH_3), 56.5(s, OCH_3), 56.4 (s, OCH_3). CIMS *m/z*: 732 $[M+H]^+$. Hyperforin nicotinate (**3**). To a solution of hyperforin (230 mg, 0.43 mmol) in dry pyridine (15 mL), excess nicotinoyl chloride (140 mg, 0.78 mmol) and a catalytic amount of DMAP (4,4 dimethylaminopyridine) were added. After stirring four hours under nitrogen, the reaction was worked up by the addition of 1N HCl (5 mL) and extraction with CH_2Cl_2 (3× 8 mL). The pooled organic phases were dried (Na_2SO_4), and the solvent was removed under vacuum. The crude material (257 mg) was purified by gravity column chromatography on silica gel (17 g, elution with petrol ether/EtOAc 17.5:2.5 (174 mg, 62.4%). Viscous oil; UV (CH_3CN): λ_{max} 273, 228, 196; $[\alpha]_D^{25}$ + 58 ($CHCl_3$, c 0.7);^{1}H NMR (400 MHz, $CDCl_3$): 9.25 (1H, br s, Ar H-2), 8.90 (bd, 3.7, Ar H-4), 8.32 (dt, 1.9, 7.9, Ar H-6), 7.51 (dd, 7.9, 4.9, Ar H-5), 5.09 (m, H32), 2× 5.08 (m, H-27+ H-22), 5.02 (br t, 5.5), 3.13 (m, H-26), 2.93 (m, H26′), 2.62 (dd, H-31), 2.32 (dd, H-31′), 1.75, 2×1.68, 2×1.63, 1.59, 1.54, 1.51 (s, H-19, H-20, H-24, H-25, H-29, H-30, H-34, H-35), 1.53 (d, 6.6, H-12), 1.06 (s, H-14), 1.03 (d, 6.6, H-13). EIMS m/z: 641 $[M]^+$.
11. 8-Chlorohyperforin (**10**). To a solution of dicyclohexylammonium hyperforinate (10 g, 14.1 mmol; containing 18% (HPLC) of the corresponding adhyperforinate) in dry dichloromethane (60 mL) *N*-chlorosuccinimide (1.89 g, 14.1 mmol, 1 mol. Equiv.) is added. The solution is stirred at room temperature and controlled by TLC (hexane–EtOAc 9:1, R_f hyperforin 0.20; R_f chloroderivative 0.80). After 30 min, the reaction was worked up by dilution with water (ca. 60 mL) and extraction with dichloromethane. The organic phase was washed with salt. $Na_2S_2O_3$ and brine, and then dried (Na_2SO_4). After removal of the solvent, the residue was purified by column chromatography on silica gel (ca. 50 mL). Elution was started with petroleum ether and was then continued with petroleum ether–EtOAc 95:5 when the chloroderivative began eluting. In this way 6.27 g of **10** was obtained as a voluminous white powder (78%). Recrystallization from warm methanol afforded an analytical sample, mp 112–119 °C (deg). $[\alpha]_D^{25}$ + 16 (CH_2Cl_2, c 0.5); IR ν^{max} (KBr): 1722, 1713, 1446, 1377, 1230, 1064, 831 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$):1.41 (m, H-4), 2.16 (m, H-5), 1.70 (m, H-5′), 2.80 (m, H-11), 1.18 (d, *J* = 7 Hz, H-12), 1.02 (d, *J* = 7 Hz, H-13), 1.06 (s, H-14), 2.01 (m, H-15), 1.06 (m, H-15′), 5.03 (m, H-17), 1.66 (br s, H-19), 1.60 (br s, H-20), 2.05 (m, H-21), 1.65 (m, H-21′), 4.76 (m, H-22), 1.66 (s, H-24), 1.52 (s, H-25), 3.18 (s, H-26), 4.96 (m, H-27), 1.63 (br s, H-29), 1.69 (br s, H-30), 2.60 (m, H-31), 5.17 (dd, *J* = 13,6, H-32), 1.66 (s, H-35). ^{13}C NMR (125 MHz, $CDCl_3$): 205.4 (s, C-1), 85.1 (s, C-2), 56.1 (s, C-3), 45.7 (d, C-4), 45.2 (t, C-5), 65.0 (s, C-6), 198.7 (s, C-7), 67.1 (s, C-8), 195.8 (s, C-9), 207.6 (s, C-10), 40.1 (d, C-11), 20.6 (q, C-12), 22.2 (q, C-13), 13.9 (q, C-14), 37.5 (t, C-15), 25.4 (t, C-16), 124.5 (d, C-17), 131.8 (s, C-18), 25.9 (q, C-19), 17.9 (q, C-20), 28.1 (t, C-21), 121.7 (d, C-22), 134.1 (s, C-23), 26.1 (q, C-24), 18.2 (q, C-25), 31.5 (t, C-26), 116.8 (d, C-27), 139.1 (s, C-28), 26.0 (q, C-29), 18.6 (q, C-30), 31.6 (t, C-31), 118.9 (d, C-32), 135.0 (s, C-33), 26.4 (q, C-34), 18.1 (q, C-35). CIMS *m/z*: 572, 570 $[M+H]^+$.
12. Octahydrohyperforin DCHA (**12**). Hyperforin DCHA (2.8 g, 5.15 mmol) was dissolved in MeOH (50 mL) and hydrogenated with Pd/C (300 mg), at room temp, and atmospheric pressure for 10 h. The heterogeneous mixture was filtered on Celite, washed with MeOH, and evaporated to dryness. The crude material was crystallized from heptane/iPrOH 2%, obtaining 2.78 g. White crystals, mp 117 °C; $[\alpha]_D^{25}$ + 19 ($CHCl_3$, c 1); IR ν^{max} (KBr): 2953, 2867, 1719, 1489, 1383, 1034, cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 2.44 (m, H-5), 2.32 (ddq, *J* = 1.9, 6.7, 6.7 Hz, H-11), 2.29 (m, H-5′), 1.17 (d, *J* = 7 Hz, H-12), 1.11 (d, *J* = 7 Hz, H-13), 0.94 (s, H-14), 0.94-0.84 (d, *J* = 8 Hz, 8 × CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): 211.0 (s, C-1), 82.0 (s, C-2), 46.8 (s, C-3), 43.2 (d, C-4), 40.7 (t, C-5), 60.5 (s, C-6), 183.0 (s, C-7), 118.6 (s, C-8), 186.0 (s, C-9), 213.0 (s, C-10), 41.1 (d, C-11), 20.8 (q, C-12), 23.3 (q, C-13), 13.4 (q, C-14), 40.7-22.0 (t, 9 × CH_2), 22.9–22.4 (q, 8× CH_3); CIMS *m/z*: 545 $[M+H]^+$.
13. *Plasmodium falciparum* assay. Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]hypoxanthine incorporation assay was used.[15] Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax II were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 μCi [^{3}H]hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated. Assays were run in duplicate and repeated once. Cytotoxicity assay. RPMI 1640 (100 μl) medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum containing 4×10^4

L-6 cells (rat skeletal myoblasts) was added to each well of a 96-well microtiter plate. After 24 h, the medium was removed from all wells and replaced by 100 μL of fresh medium in all wells except for those in row H of the plate. Fresh medium (150 μL) containing the highest drug concentration was added to wells of row H. Serial drug dilutions were prepared by transferring 50 μL from wells of row H to wells of row G. After gentle mixing, 50 μL from row G was transferred to row F, and so on. The highest concentration for the test compounds was 200 μg/mL. Seven 3-fold dilutions were used, covering a range from 200 to 0.274 μg/mL. Each drug was tested in duplicate. After 72 h of incubation, 10 μL of Alamar Blue (12.5 mg resazurin dissolved in 100 ml phosphate-buffered saline) was added to each well and the plates were incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. IC_{50} values were determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

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