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Supplementary Materials

Systematic Analysis of Clemastine, A Candidate Apicomplexan 2 Parasite-Selective Tubulin-Targeting Agent

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1. Supplementary Figures

A

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Pf  ----------MAEVAKNKNTEKLNRNEKQNDVRLTNILAAKAVADVTRTSLGPKGMDKM  49
Tg  MAAADVATKSGGSKPSAGKHDGMWAHDEKQKDIRRQNIIAAKAVADAVRTSLGPRGMDKM  60

Pf  IEDGKGGVIITNDGATILKEMAVAHPTAKMIVELSKAQDVEAGDGTTSVVVMCGSFLNVC  109
Tg  IQGPRGDVLITNDGATILNEMAVVHPVAKMMVELSKAQDVEAGDGTTSVVVLCGALLEAS  120

Pf  KSLLDKNIHCQKISESFFEASLKSEEILREMSIPIDLNDKNMLIQNAITSLNSKVVSYNS  169
Tg  QQLLDRGVHPQLIASSFLEASKQSEKILREMAVPVDLKDREKLIQIAATSLQSKVVSHNA  180

Pf  SLLAPIAVDVILKITDINKD-TNVDLNNVRIVKKLGGTIEDTEIVDGLIFTGNKISKKAQ  228
Tg  KLLAPIAVDAVMKVLPEGKDVDNVDLNDIRVVKKLGGTVDESELVKGIVMVEQKVSKRAG  240

Pf  GLKNLTQAKIGLIQFCLSLPKTDMDNTVVVKDYNSMDRLLREERLIIGKMIKKIASTGCN  288
Tg  GPSRIQNAKIGLIQFCLSAPKTDMENNIVIKDYTAMDRMLREERILIAKMVKQIAATGCN  300

Pf  LLIIQKSILRDAVNDLALDFLAKAKIMVIKDIDREDIEFISKTCNCIPVASLDYFTSDKL  348
Tg  VLLIQKSILRDAVNDLSLDYLAKAKIMVVQNIEREDVEFISRTLGCQPV-SLDHFTADKL  359

Pf  GYAENVTTE-SVGYGEIVKITGVESKNTISVLLRASNNLMLDEAERSLHDALCVVRSLIK  407
Tg  GHADLAQDEMVAGGGHIVRITGVNAKNTVTVLLRASNNLMLDETERSLHDALCVVRCLVK  419

Pf  EKAVLPGGAAPEMELSQKLYQWANTLKGSKQICVKAFSDALELIPYTLAENAGLSPLHIV  467
Tg  RRQLLPGGGAVEAELSLRLNEWARTLPGVQQLCVRMYAEALELIPYTLAENAGLSPLEIV  479

Pf  TELRNKHAEGHKYHGINIRTGTISNMIDENVIQPLLVTSTAIKLATETVMMILKIDDTVI  527
Tg  TDLKLKHAQGEKLVGINVRQGCVSSMVDINVLQPLLVSSSAVKLATEAVMMILKIDDIVM  539

Pf  CR      529
Tg  CR      541
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B

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HsA  -MPENVAPRSGATAGAAGGRGKGAYQDRDKPAQIRFSNISAAKAVADAIRTSLGPKGMDK  59
Pf   ----------M-AEVAKNKNTEKLNRNEKQNDVRLTNILAAKAVADVTRTSLGPKGMDK  48
Tg   MAAADVATKSGG-SKPSAGKHDGMWAHDEKQKDIRRQNIIAAKAVADAVRTSLGPRGMDK  59

HsA  MIQDGKGDVTITNDGATILKQMQVLHPAARMLVELSKAQDIEAGDGTTSVVIIAGSLLDS  119
Pf   MIEDGKGGVIITNDGATILKEMAVAHPTAKMIVELSKAQDVEAGDGTTSVVVMCGSFLNV  108
Tg   MIQGPRGDVLITNDGATILNEMAVVHPVAKMMVELSKAQDVEAGDGTTSVVVLCGALLEA  119

HsA  CTKLLQKGIHPTIISESFQKALEKGIEILTDMSRPVELSDRETLLNSATTSLNSKVVSQY  179
Pf   CKSLLDKNIHCQKISESFFEASLKSEEILREMSIPIDLNDKNMLIQNAITSLNSKVVSYN  168
Tg   SQQLLDRGVHPQLIASSFLEASKQSEKILREMAVPVDLKDREKLIQIAATSLQSKVVSHN  179

HsA  SSLLSPMSVNAVMKVIDPATA-TSVDLRDIKIVKKLGGTIDDCELVEGLVLTQKV---SN  235
Pf   SSLLAPIAVDVILKITDINKD-TNVDLNNVRIVKKLGGTIEDTEIVDGLIFTGNKISKKA  227
Tg   AKLLAPIAVDAVMKVLPEGKDVDNVDLNDIRVVKKLGGTVDESELVKGIVMVEQKVSKRA  239

HsA  SGITRVEKAKIGLIQFCLSAPKTDMDNQIVVSDYAQMDRVLREERAYILNLVKQIKKTGC  295
Pf   QGLKNLTQAKIGLIQFCLSLPKTDMDNTVVVKDYNSMDRLLREERLIIGKMIKKIASTGC  287
Tg   GGPSRIQNAKIGLIQFCLSAPKTDMENNIVIKDYTAMDRMLREERILIAKMVKQIAATGC  299

HsA  NVLLIQKSILRDALSDLALHFLNKMKIMVIKDIEREDIEFICKTIGTKPVAHIDQFTADM  355
Pf   NLLIIQKSILRDAVNDLALDFLAKAKIMVIKDIDREDIEFISKTCNCIPVASLDYFTSDK  347
Tg   NVLLIQKSILRDAVNDLSLDYLAKAKIMVVQNIEREDVEFISRTLGCQPV-SLDHFTADK  358

HsA  LGSAELAEEVNLNGSGKLLKITGCASPGKTVTIVVRGSNKLVIEEAERSIHDALCVIRCL  415
Pf   LGYAENVTTE-SVGYGEIVKITGVESK-NTISVLLRASNNLMLDEAERSLHDALCVVRSL  405
Tg   LGHADLAQDEMVAGGGHIVRITGVNAK-NTVTVLLRASNNLMLDETERSLHDALCVVRCL  417

HsA  VKKRALIAGGGAPEIELALRLTEYSRTLSGMESYCVRAFADAMEVIPSTLAENAGLNPIS  475
Pf   IKEKAVLPGGAAPEMELSQKLYQWANTLKGSKQICVKAFSDALELIPYTLAENAGLSPLH  465
Tg   VKRRQLLPGGGAVEAELSLRLNEWARTLPGVQQLCVRMYAEALELIPYTLAENAGLSPLE  477

HsA  TVTELRNRHAQGEKTAGINVRKGGISNILEELVVQPLLVSVSALTLATETVRSILKIDDV  535
Pf   IVTELRNKHAEGHKYHGINIRTGTISNMIDENVIQPLLVTSTAIKLATETVMMILKIDDT  525
Tg   IVTDLKLKHAQGEKLVGINVRQGCVSSMVDINVLQPLLVSSSAVKLATEAVMMILKIDDI  537

HsA  VNTR   539
Pf   VICR   529
Tg   VMCR   541
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C

Pairwise TriC-δ comparisons			
	Plasmodium	*Toxoplasma*	Human
Plasmodium	100	80.3% S	73.9% S
Toxoplasma	63.5% I	100	77.8% S
Human	57.0% I	57.7% I	100

Figure S1. Clustal omega alignments of the tubulin chaperone TRiC subunit from *Toxoplasma* (Tg) and *Plasmodium falciparum* (Pf) (**A**) or *Toxoplasma*, *Plasmodium* and human (Hs) proteins (**B**). Amino acids are colored according to their physicochemical properties: small and hydrophobic residues are red, acidic are blue, basic are magenta, and the remaining amino acids are colored green. (**C**) Pairwise comparisons performed with EMBOSS Needle [47] indicate that *Toxoplasma* and *Plasmodium* TRiC-δ proteins have greater identity (I) and similarity (S) than either parasite protein.

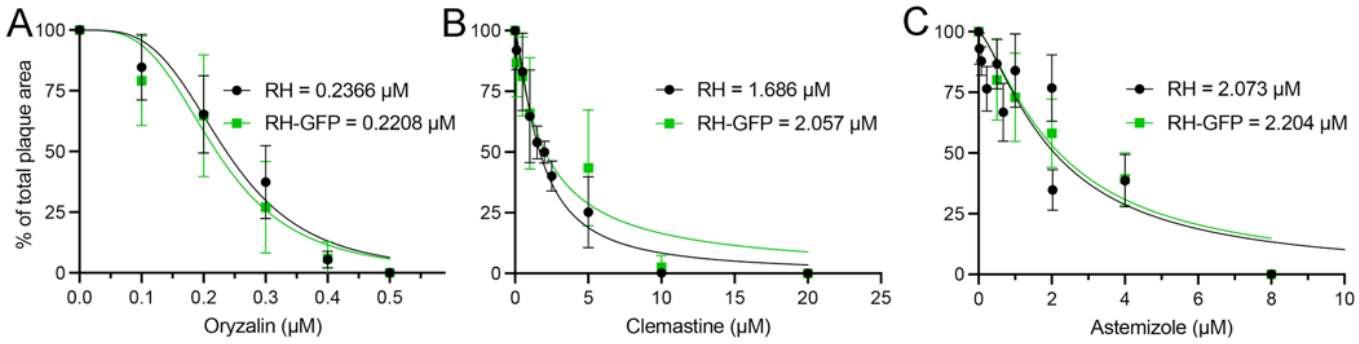

Figure S2. Plaque-based EC_{50} assays measure inhibition of lytic growth. The relationship between plaque area and drug concentration is graphed to determine the 50% inhibitory concentration with values normalized to vehicle control cultures. The values for oryzalin (**A**), clemastine (**B**) and astemizole (**C**) show that all exhibit antiparasitic activity in the micromolar range and are comparably active at inhibiting both RH strain parasites and a GFP-expressing RH-derived line. All results represent the average of at least 9 readings (3 biological replicates, each with 3 technical replicates) ± standard deviation.

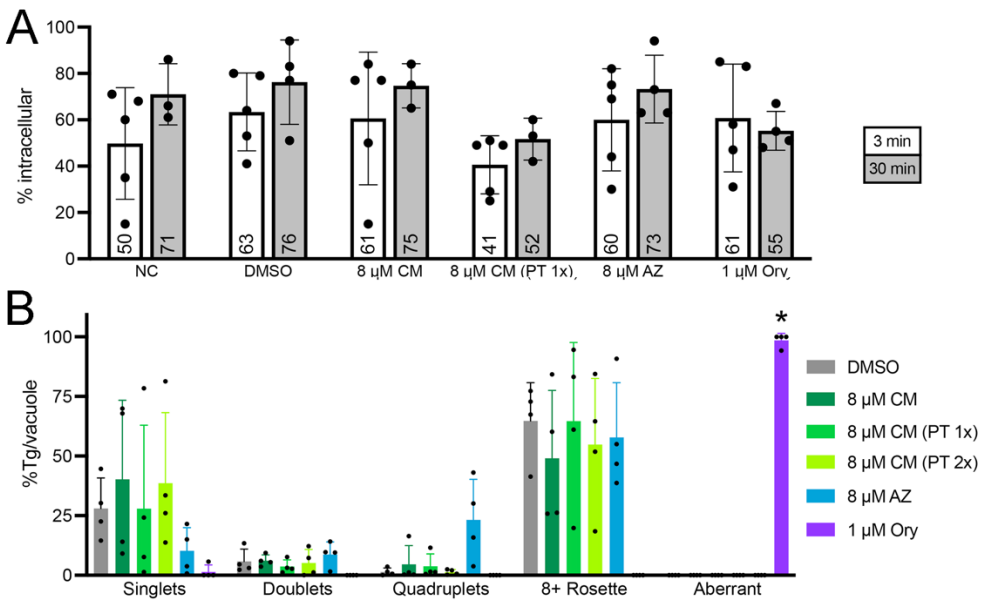

Figure S3. Lytic growth consists of invasion, replication, and egress. (**A**) GFP-expressing RH strain tachyzoites are added to HFF cells in control or drug-containing media for 3-30 minutes. After fixation subsequent antibody staining without permeabilization distinguishes intracellular tachyzoites from extracellular (host cell attached) parasites, which are detected by staining for the externally exposed SAG-1 surface antigen detected with a red-fluorescing secondary antibody. Statistical analyses were run using an ordinary two-way ANOVA. The results represent the average of 30 readings (3 biological replicates with 10 fields of view for each treatment) ± standard deviation. (**B**) Intracellular *Toxoplasma* parasites within membrane-bound vacuoles replicate by endodyogeny. RH strain tachyzoites double every 60 minutes and replication within a vacuole is largely synchronous, leading to exponential expansion of parasites. Live imaging of GFP-expressing tachyzoites permits detection of differences in replication rate and overt changes in parasite morphology or differentiation state. No delays in replication are detected at 36 hours for clemastine and astemizole; oryzalin treated parasites rapidly transform to aberrant forms. Data are displayed as the fraction of total vacuoles/field that contain one, two, four, or eight or more (8+) parasites. Statistical analyses were run using a two-way ANOVA. The results represent the average of 40 readings (4 biological replicates with 10 fields of view for each treatment) ± standard deviation.

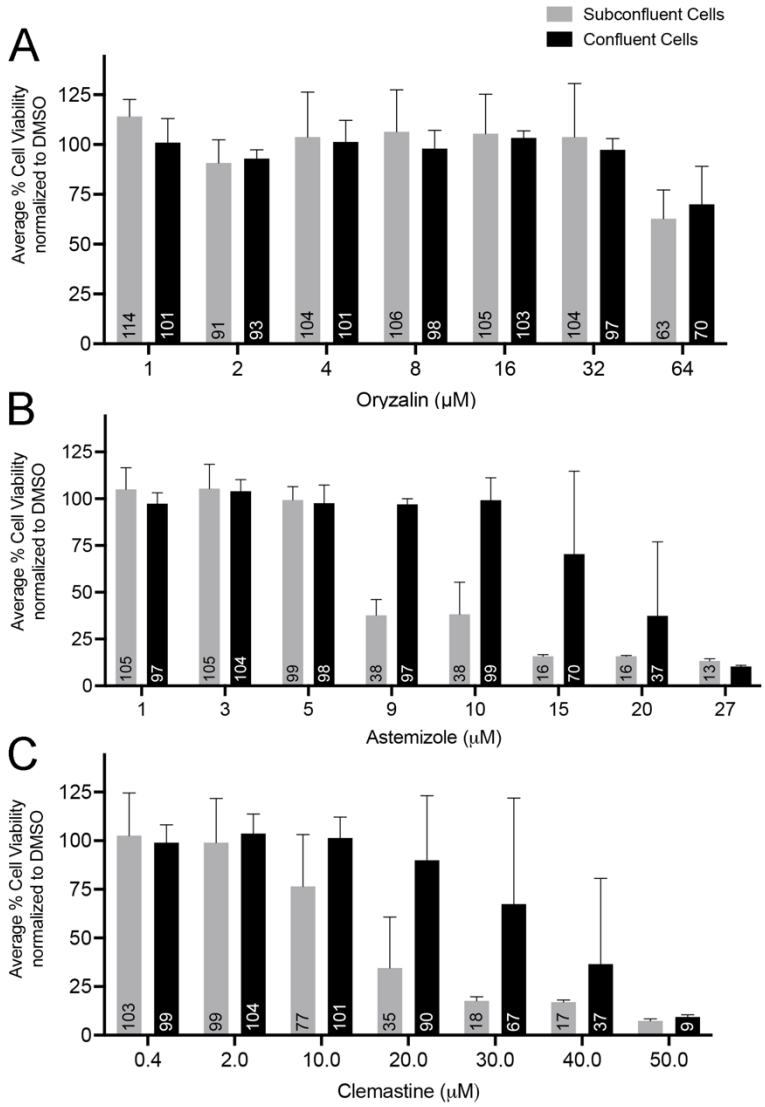

Figure S4. MTT assay-based quantification of compound toxicity to human fibroblasts. Because replicating human fibroblasts are likely to be more sensitive to compounds than contact-inhibited confluent monolayers, we evaluated the effects of oryzalin (**A**), astemizole (**B**), and clemastine (**C**) on both replicating (light gray) and confluent (dark gray) monolayers of HFF cells. In all compounds, subconfluent cultures showed greater compound sensitivity. All results represent the average of at least 9 readings (3 biological replicates, each with 3 technical replicates) ± standard deviation. .