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Aryl Quinazolinone Derivatives as Novel Therapeutic Agents against Brain-Eating Amoebae

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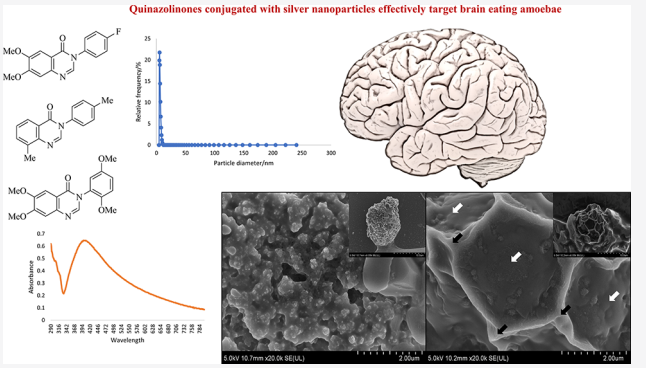
ABSTRACT: *Naegleria fowleri* and *Balamuthia mandrillaris* are protist pathogens that infect the central nervous system, causing primary amoebic meningoencephalitis and granulomatous amoebic encephalitis with mortality rates of over 95%. Quinazolinones and their derivatives possess a wide spectrum of biological properties, but their antiamoebic effects against brain-eating amoebae have never been tested before. In this study, we synthesized a variety of 34 novel arylquinazolinones derivatives (Q1−Q34) by altering both quinazolinone core and aryl substituents. To study the antiamoebic activity of these synthetic arylquinazolinones, amoebicidal and amoebistatic assays were performed against *N. fowleri* and *B. mandrillaris*. Moreover, amoebae-mediated host cells cytotopathogenicity and cytotoxicity assays were performed against human keratinocytes cells *in vitro*. The results revealed that selected arylquinazolinones derivatives decreased the viability of *B. mandrillaris* and *N. fowleri* significantly ($P < 0.05$) and reduced cytopathogenicity of both parasites. Furthermore, these compounds were also found to be least cytotoxic against HaCat cells. Considering that nanoparticle-based materials possess potent *in vitro* activity against brain-eating amoebae, we conjugated quinazolinones derivatives with silver nanoparticles and showed that activities of the drugs were enhanced successfully after conjugation. The current study suggests that quinazolinones alone as well as conjugated with silver nanoparticles may serve as potent therapeutics against brain-eating amoebae.



KEYWORDS: *Brain-eating amoeba, antiamoebic, medicinal chemistry, quinazolinone, nanoparticles*

INTRODUCTION

Naegleria fowleri and *Balamuthia mandrillaris* are known as brain-eating amoebae that infect the central nervous system (CNS) and cause primary amoebic meningoencephalitis (PAM) and granulomatous amoebic encephalitis (GAE), respectively.[1,2] Infection of the CNS with free-living amoebae almost always proves to be fatal with a mortality rate of over 95%.[3−6] Despite the high mortality rate, currently there is no single effective drug available against these infections. Drugs such as amphotericin B, miconazole, rifampin, chloramphenicol, ornidazole, dexamethasone, ceftriaxone, fluconazole, miltefosine, azithromycin, 5-fluorocytosine, pentamidine, sulfadiazine, clarithromycin, trifluoperazine, ketoconazole, flucytosine, itraconazole, metronidazole, thioridazine, and artesunate have shown efficacy against *N. fowleri* and *B. mandrillaris*.[5] Specifically, a combination of amphotericin B, fluconazole, rifampin, azithromycin, dexamethasone, and miltefosine is effective against PAM due to *N. fowleri*, while a combination of pentamidine, sulfadiazine, flucytosine, fluconazole, azithromycin, and miltefosine is effective against GAE due to *B. mandrillaris*.[5−8] However, the optimal regimen against these pathogens has not been established and still a high dosage of a mixture of drugs is recommended as treatment against PAM and GAE.[7,8] These drugs generally lack specificity and exhibit high host cell cytotoxicity; therefore, there is an urgent need for the development of efficient and safer treatment options against these devastating infections.[2]

Quinazolinones and their derivatives are among one of the most important heterocycles in medicinal chemistry as they possess a wide spectrum of biological properties.[9] It has been reported that quinazolinones demonstrate antifungal activities against organisms such as *Aspergillus niger*, *Candida albicans*, and *Aspergillus clavatus*.[10−12] Moreover, quinazolinones also possess antibacterial activity against several bacteria, including *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*.[10,12−16] Furthermore, the anticancer properties

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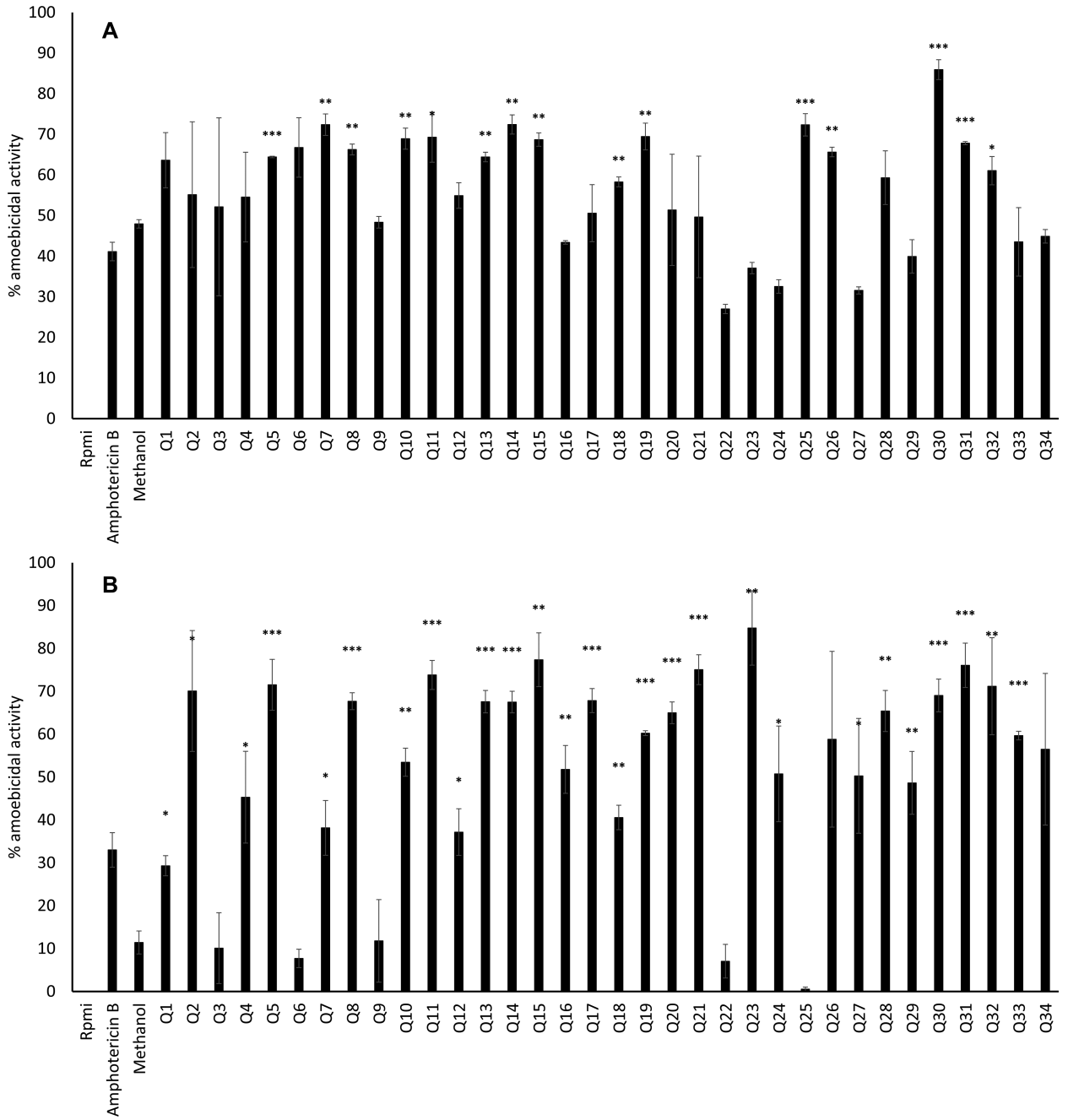

Figure 1. (A) Amoebicidal activities of quinazolinones were determined. Briefly, *B. mandrillaris* was incubated with various quinazolinones for 24 h and viability was then determined using Trypan blue as described in the Methods section. The results revealed that quinazolinones caused a reduction in the number of viable *B. mandrillaris* cells. The results are representative of at least three independent experiments performed in duplicate. The data are presented as the mean ± standard error (*P < 0.05, **P < 0.01, ***P < 0.001 using two sample t test; two tailed distribution). (B) Amoebicidal activities of quinazolinones were determined. Briefly, *N. fowleri* was incubated with various quinazolinones for 24 h and viability was then determined using Trypan blue as described in Methods. The results revealed that quinazolinones caused a reduction in the number of viable *N. fowleri* cells. The results are representative of at least three independent experiments performed in duplicate. The data are presented as the mean ± standard error (*P < 0.05, **P < 0.01, ***P < 0.001 using two sample t test; two tailed distribution).

of quinazolinones have been evaluated, and it was reported that the compounds exhibit potent anticancer activity at low concentrations.[17−19] Quinazolinones also exhibit noteworthy effects against viruses, such as vaccinia virus, Pox viruses, influenza A virus, and human immunodeficiency virus.[16,20,21] Several quinazoline and quinazolinone derivatives have also been reported to exhibit antiparasitic activities such as antileishmanial,[22] antiplasmodial,[23] antiacanthamoebic,[24] antitrypanosoma,[25] and antitoxoplasma activities.[26] However, despite their broad range of biological properties, quinazolinones have not been tested against *N. fowleri* and *B. mandrillaris*.

Considering the potent activity of quinazolinones against eukaryotic fungi and parasites, we hypothesize that quinazolinones possess antiamoebic activity against brain-eating amoebae *N. fowleri* and *B. mandrillaris*. A library of 34 aryl quinazolin-4(3*H*)-one derivatives was synthesized by multicomponent reactions, and these compounds were thoroughly

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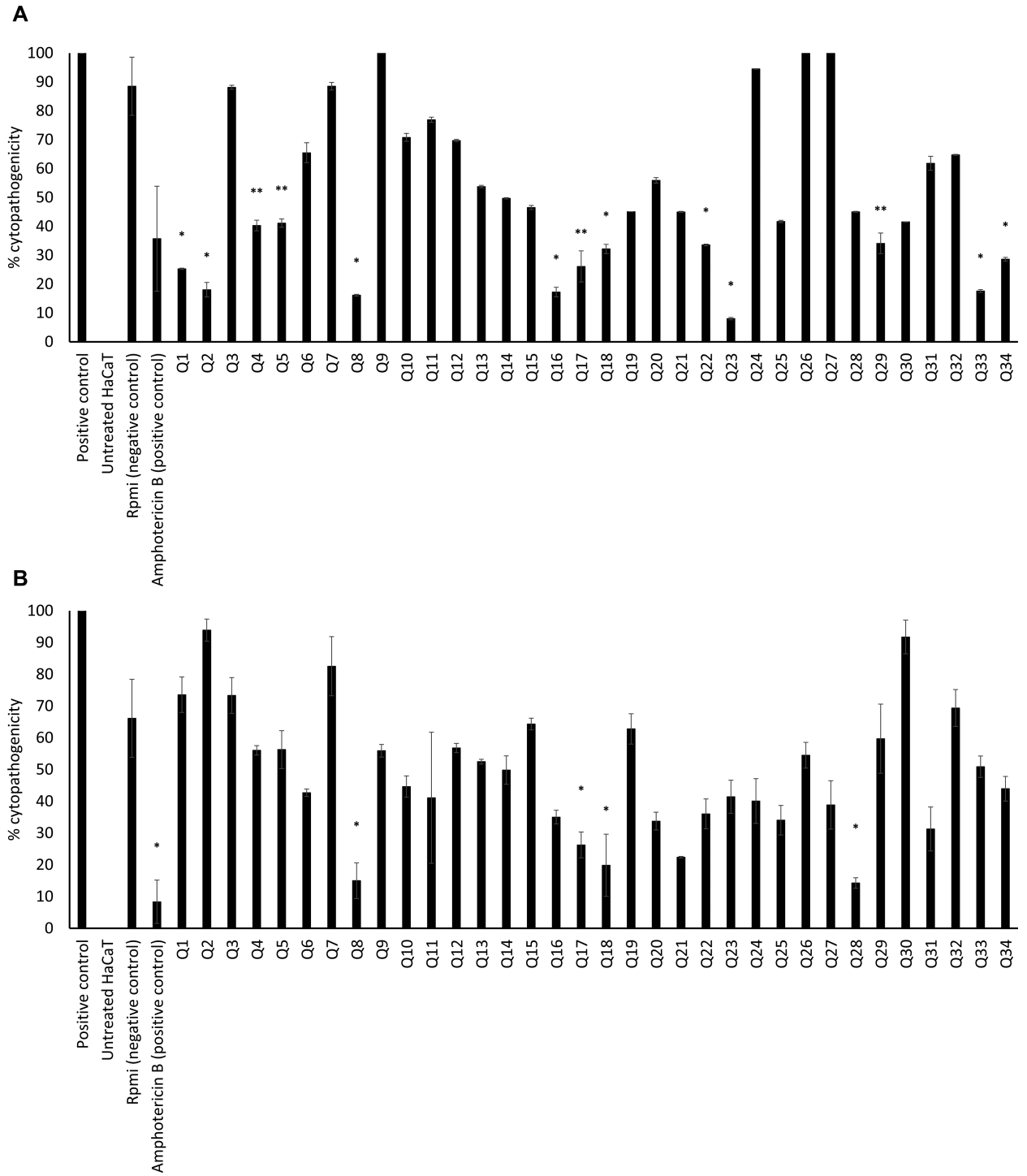

Figure 2. (A) Representative effects of quinazolinones on *B. mandrillaris*-mediated HeLa cell cytotoxicity. Briefly, amoebae (5×10^5 amoebae/0.5 mL) were incubated with 100 μg/mL of quinazolinones and then added to HeLa cells monolayers for 48 h at 37 °C in a 5% CO_2 incubator as described in the Methods section. The results showed that some quinazolinones inhibited parasite-mediated host cell damage. The data are presented as the mean ± standard error (*$P < 0.05$, **$P < 0.01$ using two sample *t* test; two tailed distribution). (B) Representative effects of quinazolinones on *N. fowleri* mediated HeLa cell cytotoxicity. Briefly, amoebae (5×10^5 amoebae/0.5 mL) were incubated with 100 μg/mL of quinazolinones and then added to HeLa cells monolayers for 48 h at 37 °C in a 5% CO_2 incubator as described in the Methods section. The results showed that some quinazolinones inhibited parasite-mediated host cell damage. The data are presented as the mean ± standard error (*$P < 0.05$ using two sample *t* test; two tailed distribution).

characterized by standard spectroscopic techniques including nuclear magnetic resonance spectroscopy and mass spectrometry as reported previously.[24] These compounds were tested against *N. fowleri* and *B. mandrillaris* for their amoebicidal properties, their amoebistatic properties, and their ability to inhibit amoebae-mediated host cell damage. Moreover, some of these compounds were further conjugated with silver nanoparticles as representatives to determine if the anti-amoebic activity is enhanced by nanoparticles conjugation. For the first time, we showed that nanoparticle-coated quinazoli-

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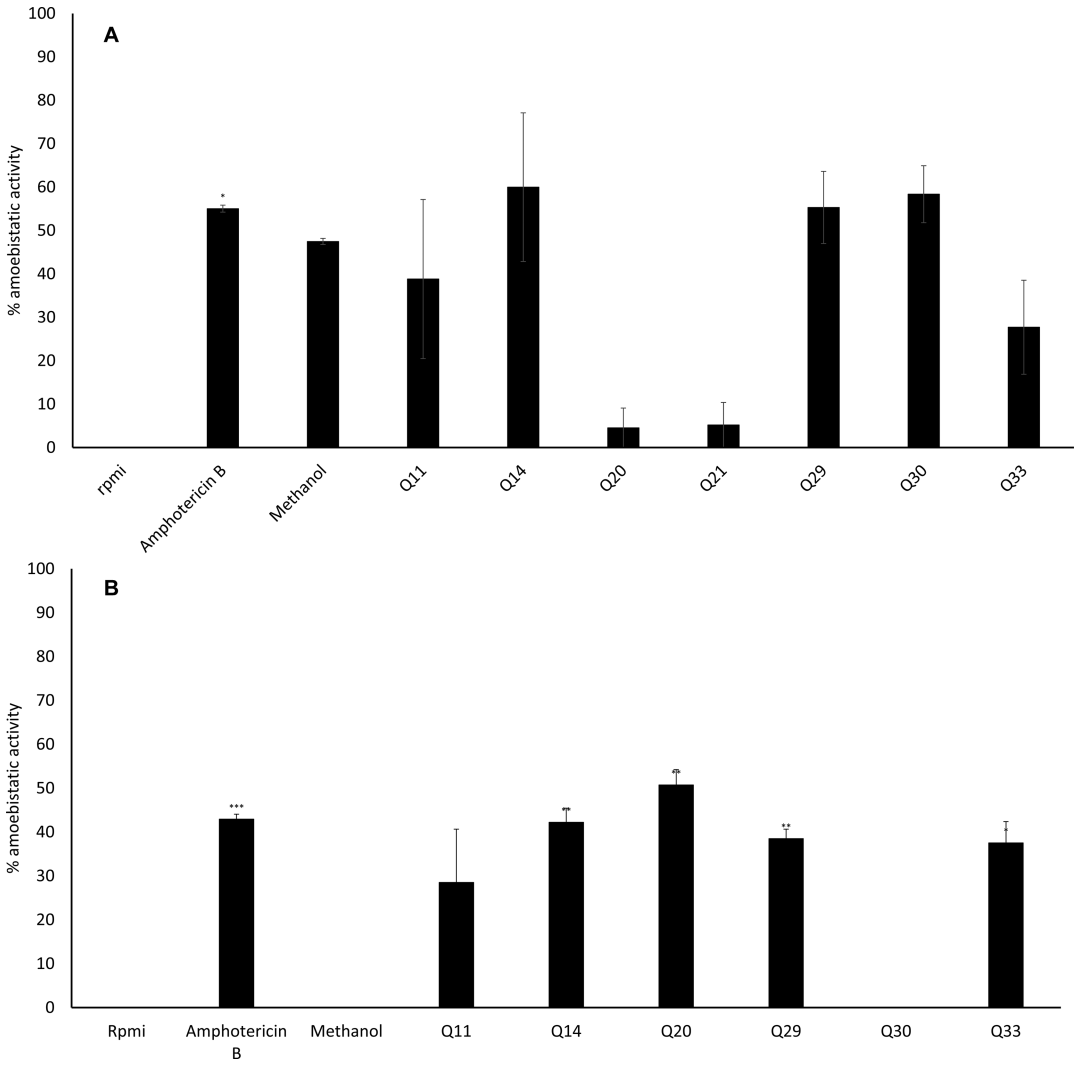

Figure 3. (a) Amoebistatic activities of quinazolinones were determined. Briefly, *B. mandrillaris* was incubated with various quinazolinones onto a monolayer of feeder cells for 24 h and viability was then determined using Trypan blue as described in the Methods section. The results revealed that quinazolinones caused a reduction in the growth of *B. mandrillaris* cells. The results are representative of at least three independent experiments performed in duplicates. The data are presented as the mean ± standard error. (b) Amoebistatic activities of quinazolinones were determined. Briefly, *N. fowleri* was incubated with various quinazolinones onto a monolayer of feeder cells for 24 h and viability was then determined using Trypan blue as described in the Methods section. The results revealed that quinazolinones caused a reduction in the growth of *N. fowleri* cells. The results are representative of at least three independent experiments performed in duplicate. The data are presented as the mean ± standard error (*$P < 0.05$, **$P < 0.01$ using two sample t test; two tailed distribution).

nones exhibited potent in vitro activity against brain-eating amoebae and conjugation of drugs with silver nanoparticles significantly enhanced activity of several derivatives against *N. fowleri* and *B. mandrillaris*. Furthermore, nanoparticle-coated quinazolinones inhibited parasite-mediated human cell cytotoxicity. Based on the results presented in the present study, quinazolinones can be considered as potential candidate for future drug development studies against brain-eating amoebae.

RESULTS AND DISCUSSION

Primary amoebic meningoencephalitis and granulomatous amoebic encephalitis are fatal brain infections caused by *N. fowleri* and *B. mandrillaris* respectively.[7,8] The high mortality rates despite the existing treatment options suggest the need for novel drugs. Quinazolinones have previously been shown to demonstrate a wide spectrum of biological properties.[9] Quinazolinones have been shown to act using various mechanisms, including inhibition of deoxyribonucleic acid (DNA) synthesis and inhibition of cell wall synthesis in bacteria, while inducing apoptosis, inhibition of DNA repair and attachment to DNA in cancer cells.[14−26] Therefore, we hypothesized that these compound classes may possess antiamoebic activities and tested them against *N. fowleri* and *B. mandrillaris* for their amoebicidal properties, amoebistatic properties and their ability to inhibit amoebae-mediated host cell damage.

Several Quinazolinones Showed Amoebicidal Activity against *B. mandrillaris* and *N. fowleri*. Amoebicidal assays were performed to determine the ability of quinazolinones to kill the amoebae. The use of 100 μg/mL quinazolinones caused a reduction in the number of viable *B. mandrillaris* as shown in Figure 1A. Q5, Q7, Q10, Q11, Q13−Q15, Q18, Q19, Q25, Q26, and Q30−Q32 showed statistically significant effects against *B. mandrillaris* when compared to the solvent control ($P < 0.05$ using two sample t test; two tailed distribution). The compound that resulted in the lowest

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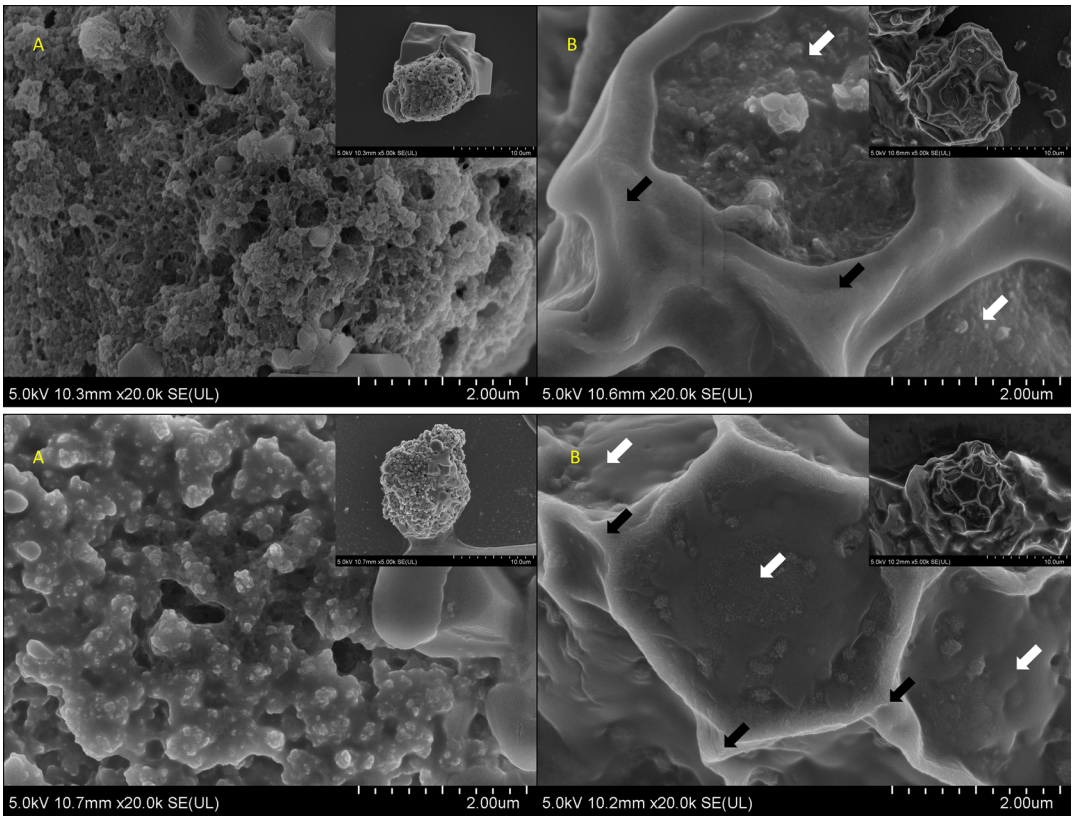

Figure 4. (Top) FE-SEM images of *B. mandrillaris* treated with quinazolinones. Amoebae treated with quinazolinones were fixed on glass coverslips by using glutaraldehyde. Following fixation, images were recorded using a field-emission scanning electron microscope (Hitachi SU8010). (A) Untreated (B) treated with 100 μg/mL Q15. (Bottom) FE-SEM images of *N. fowleri* treated with quinazolinones. Amoebae treated with quinazolinones were fixed on glass coverslips by using glutaraldehyde. Following fixation, images were recorded using a field-emission scanning electron microscope (Hitachi SU8010). (A) Untreated (B) treated with 100 μg/mL Q5.

number of viable *B. mandrillaris* is Q30, resulting in 86% cell death. Moreover, selected quinazolinones resulted in a reduction of the number of viable *N. fowleri* cells as shown in Figure 1B. These include Q1, Q2, Q4, Q5, Q7, Q8, Q10−Q12, Q14−Q21, Q23, Q24, and Q27−Q33. The drugs with the highest amoebicidal activities against *N. fowleri* were Q23 with a reduction of viable cells by 85%.

Several Quinazolinones Reduced the Cytopathogenicity of *B. mandrillaris* and *N. fowleri* against Human Cells. Assays were performed to assess the effect of the drugs on the cytopathogenicity of the amoebae against human cells. A concentration of 100 μg/mL of Q1, Q2, Q4, Q5, Q8, Q16−Q18, Q22, Q23, Q29, Q33, and Q34 resulted in a statistically significant decrease in the cytopathogenicity of *B. mandrillaris* against HeLa cells when compared to untreated *B. mandrillaris* as shown in Figure 2A ($P < 0.05$). As compared to cytopathogenicity of 69% caused by untreated amoebae, *B. mandrillaris* treated with Q23 and Q8 had a cytopathogenicity of 8% and 16%, respectively. A decrease was also observed in the cytopathogenicity of *N. fowleri* treated with 100 μg/mL of selected quinazolinones as shown in Figure 2B. Q8, Q17, Q18, and Q28 reduced the cytopathogenicity of the amoeba when compared to the untreated *N. fowleri* which exhibited a cytopathogenicity of 66%. *N. fowleri* treated with Q28 had a cytopathogenicity of 14% as compared to 66% for the untreated amoebae.

Various Quinazolinones Inhibited the Growth of *B. mandrillaris* and *N. fowleri*. Selected quinazolinones were tested for amoebistatic activity. A concentration of 100 μg/mL of Q11, Q14, Q29, Q30, and Q33 inhibited the growth of *B. mandrillaris* but was not statistically significant when compared to the solvent control which inhibited the growth of the amoebae by 47.5%. As shown in Figure 3A, Q14 and Q30 inhibited the growth of the amoeba by 60% and 58%, respectively, when compared to untreated *B. mandrillaris*. A decrease in the growth was also observed in *N. fowleri* treated with 100 μg/mL of some quinazolinones as shown in Figure 3B. Q14, Q20, Q29, and Q33 are the compounds that were able to inhibit the growth of the amoebae. Q20 inhibited the growth of *N. fowleri* by 51%, while Q14 inhibited the growth of the amoebae by 42% when compared with untreated amoebae.

Scanning Electron Microscopy. The effects of the quinazolinones on the morphology of the amoebae were observed using field-emission scanning electron microscopy (FE-SEM) at 5×, 10^3×, and 2 × 10^4×. A difference in morphology was observed between the untreated *B. mandrillaris* cells and the amoebae treated with Q15 as shown in Figure 4, top. The untreated amoebae appeared as a circular mass with an uneven surface. While the treated cells were also circular in shape, but they appeared wrinkled and the formation of craters (white arrows) surrounded by a thick film (black arrows) was observed. A difference in morphology was also observed between the untreated and treated *N. fowleri* as shown in Figure 4, bottom. The untreated *N. fowleri* appeared as a circular mass with an uneven surface, while the amoebae treated with Q5 appeared as a shrunken circular mass with the formation of craters with a comparatively smooth surface (white arrows) surrounded by a thick border (black arrows).

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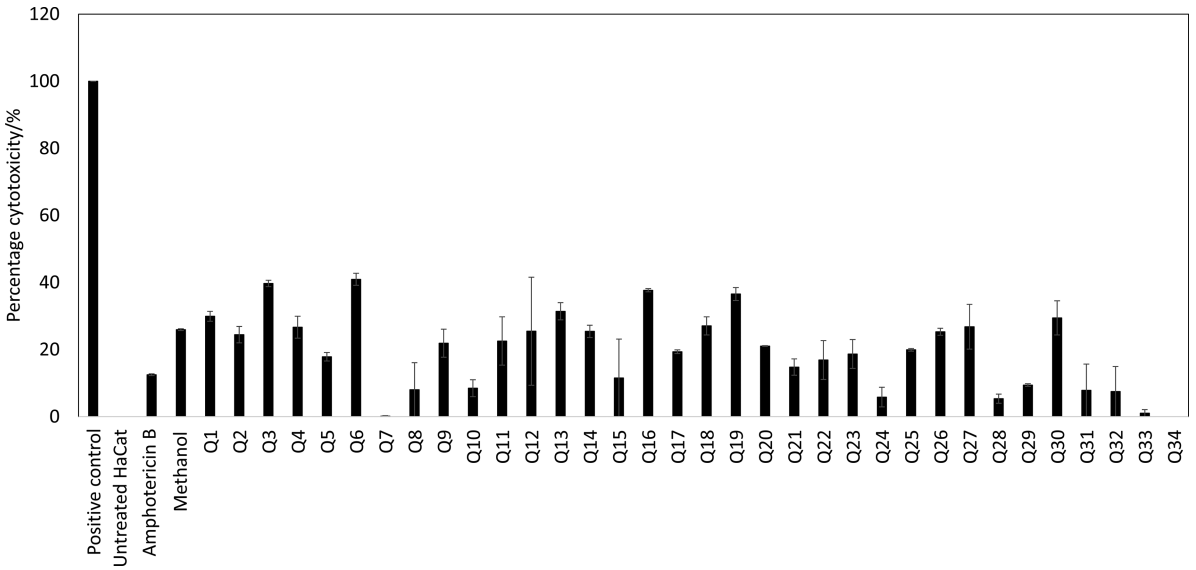

Figure 5. Cytotoxic effects of quinazolinones against host cells. Briefly, 100 μg/mL of quinazolinones were incubated with HaCaT cells monolayers for 24 h at 37 °C in a 5% CO_2 incubator as described in Materials and Methods. The results showed that quinazolinones have limited host cell damage. The data are presented as the mean ± standard error.

Quinazolinones Exhibited Limited Cytotoxicity against Human Cells. The cytotoxic effects of the quinazolinones on human epithelial cells (HaCaT) was determined by lactate dehydrogenase assay. The results indicate that most of the quinazolinones had a cytotoxicity lower than 25% at a concentration of 100 μg/mL as shown in Figure 5. Q34 had no cytotoxic activity against HaCaT while Q7 and Q33 only expressed limited (less than 5%) cytotoxicity against the cell line.

Quinazolinones were Successfully Conjugated with Silver Nanoparticles. Formation of a characteristic band at around 420 nm, which suggests the formation of the silver nanoparticles was observed for six of the quinazolinone conjugated with silver nanoparticles (Supporting Information). Peaks can be observed at below 400 nm, suggesting formation of nanoparticles, while the zeta potential of quinazolinones after conjugation with silver nanoparticles showed peaks at low millivolts suggesting stability of nanoparticles (Supporting Information).

All the quinazolinones conjugated silver nanoparticles gave an average size of less than 50 nm. Notably, Ag-Q13 has an average size of 6 nm and Ag-Q15 has an average size of 5 nm. The zeta potential of quinazolinones conjugated with silver nanoparticles was found to be averaged in the range of −30 to −22 mV, indicating stability of the small particles.

Conjugation of Quinazolinones with Silver Nanoparticles Enhanced the Activity of Q24 against *B. mandrillaris* against *N. fowleri*. The activity of quinazolinones before and after conjugation was investigated. Q13 activity was enhanced from no visible amoebicidal activity before conjugation to causing 40% amoebicidal effect against *B. mandrillaris* while activity of Q24 was increased from 14% to an amoebicidal effect of 42% as shown in Figure 6A. A 41% decrease in viability of *B. mandrillaris* was observed for Q34 conjugated with silver nanoparticles, while 25% activity was observed for the drug before conjugation. The activity of Q12, Q13, Q15, Q18, Q24, and Q34 was investigated before and after conjugation with silver nanoparticles against *N. fowleri*. The activity of Q24 was enhanced from 37% to 53% against *N. fowleri* after conjugation with silver nanoparticles (Figure 6B).

Structure−Activity Relationship. Q8 and Q18 showed amoebicidal activities against both amoebae and also reduced their cytopathogenicity. Q30 and Q23 showed the most amoebicidal and Q14 and Q20 amoebistatic activities against *B. mandrillaris* and *N. fowleri*, respectively. Q23 and Q28 are the quinazolinones that were able to reduce the cytopathogenicity of *B. mandrillaris* and *N. fowleri*, respectively. Q8 possesses an iodine, while Q18 possesses a methyl group at position 2 on the benzene ring attached to the quinazoline. Q14 and Q28 contain a methoxy group, while Q23 possesses a butyl group at position 4 on the benzene ring attached to the quinazoline. Q30 possesses two methoxy groups, while Q20 possesses a methyl and a chlorine group at position 2 and 5 on the benzene ring attached to the quinazoline. The results suggest that quinazolinones that exhibited activity against *B. mandrillaris* and *N. fowleri* possess chlorine, methyl, or methoxy groups. Previous studies have shown that the presence of halogen atoms and lipophilic electron withdrawing groups on quinazolinones enhances their antimicrobial activity.[27−31] It has also been shown that the antimicrobial activity of quinazolinones can be enhanced by the presence of methyl groups.[27] Moreover, the enhancement of the antimicrobial activity of quinazolinones by the presence of methoxy groups has also been reported.[9,29] Furthermore, it has been reported that the varying positions and types of substituents on the quinazolinones may alter the biological properties of the drugs.[32]

Small sizes of nanoparticles make them efficient carriers which enhances both pharmacokinetics and pharmacodynamics of drugs.[33] Furthermore, it has been previously shown that conjugation of drugs with silver nanomaterials may enhance activity of drugs against brain-eating amoebae.[30] Selected quinazolinones were therefore conjugated with silver nanoparticles and tested for amoebicidal activities against *N. fowleri* and *B. mandrillaris*. It has been previously reported that quinazolinones conjugated with silver possess potent antimicrobial activities.[34] This is consistent with our results which

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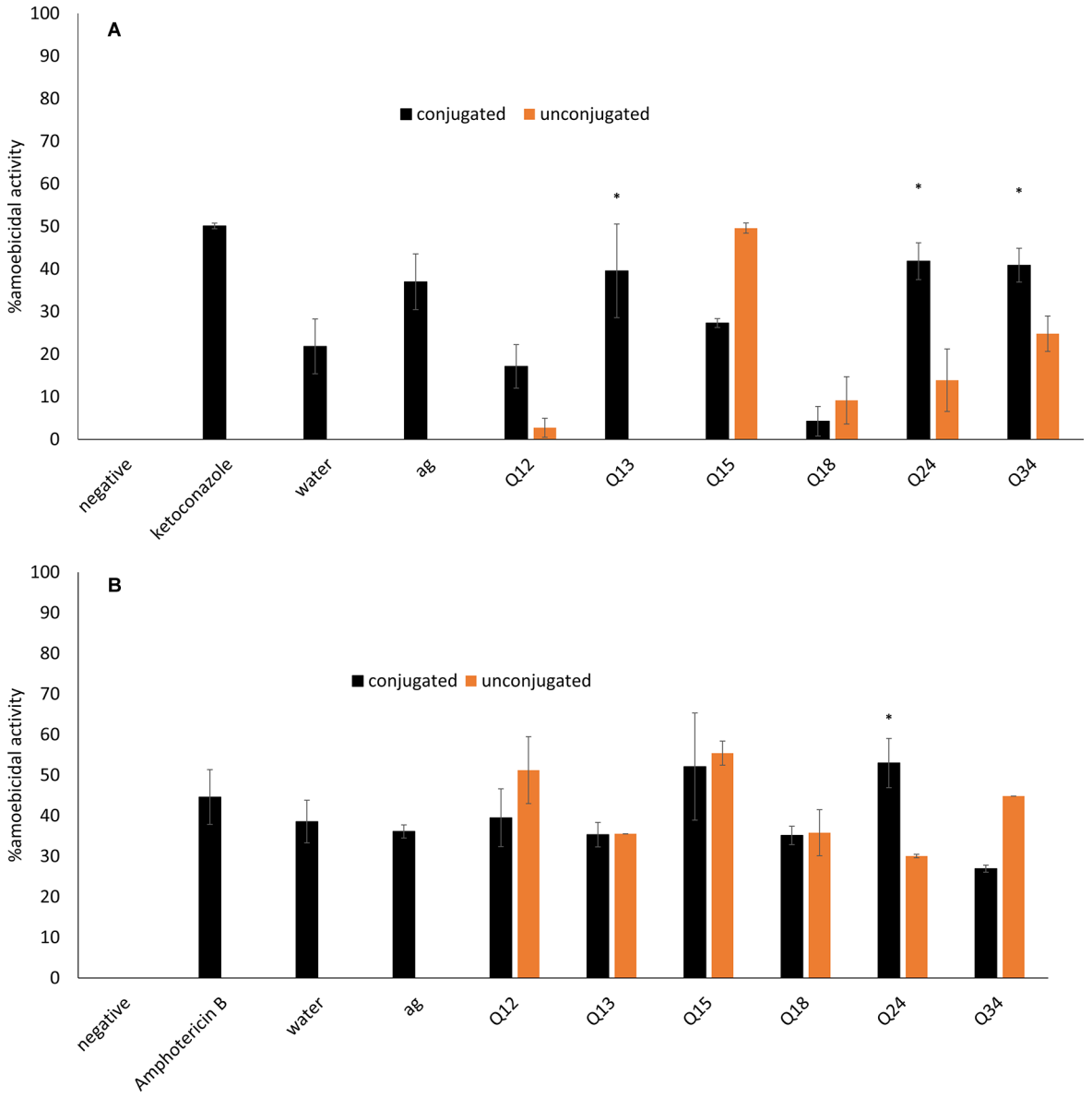

Figure 6. (A) Amoebicidal activities of quinazolinones were determined. Briefly, *B. mandrillaris* was incubated with various quinazolinones for 24 h, and viability was then determined using Trypan blue as described in the Methods section. The results show the effect of quinazolinones before and after conjugation on *B. mandrillaris* cells. The results are representative of at three independent experiments performed in duplicates. The data are presented as the mean ± standard error (*$P < 0.05$ using two sample *t* test; two tailed distribution). (B) Amoebicidal activities of quinazolinones were determined. Briefly, *N. fowleri* was incubated with various quinazolinones for 24 h, and viability was then determined using Trypan blue as described in the Methods section. The results show effect of quinazolinones before and after conjugation on *N. fowleri* cells. The data are presented as the mean ± standard error (*$P < 0.05$ using two sample *t* test; two tailed distribution).

suggest that the conjugation of quinazolinones with silver nanoparticles enhances their in vitro activity against both *B. mandrillaris* and *N. fowleri*.

In conclusion, several quinazolinones, mainly those possessing halogen, methoxy, methyl, and butyl substituents, exhibited potent antiamoebic activity. The antiamoebic effects of quinazolinones are also shown to be enhanced after conjugation with silver nanoparticles. This suggest that quinazolinones hold promise for drug development against *B. mandrillaris* and *N. fowleri*. However, the mechanism of action of the drugs, as well as the activity of the drugs *in vivo*, is not clear and will be investigated in future studies.

METHODS

Synthesis of Aryl Quinazolines. A total of thirty four aryl quinazolinones were synthesized via one pot reaction of 2-amino-3-methylbenzoic acid or 2-amino-4,5-dimethoxybenzoic acid (1 mmol) with triethoxymethane (3 mmol) and varying substituted anilines (1 mmol) refluxed in acidic medium using acetic acid for 8−12 h to afford 15 3-aryl-8-methylquinazolin-4(3*H*)-ones and 19 3-aryl-6,7-dimethoxyquinazolin-4(3*H*)-ones as described previously.[22] After the complete consumption of starting materials monitored by thin layer chromatography analysis, the reaction mixture was poured in water, precipitates formed were filtered, and the mixture was thoroughly washed with ultrapure water and dried under vacuum. The crude solid obtained was crystallized by ethanol. The structural elucidation and purity were determined by nuclear magnetic resonance spectroscopy (^{1}H NMR), electron impact mass spectrometry (EI-MS), and elemental analysis. The structure and molecular details of all 34

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Table 1. Table Shows the Different Quinazolinones That Were Tested against the Amoebae[a]

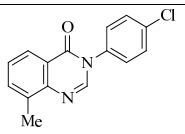
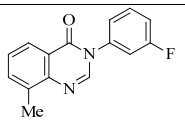
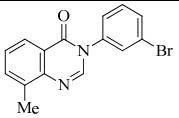
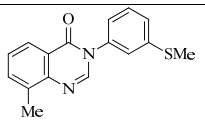
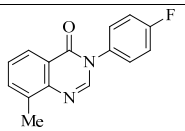
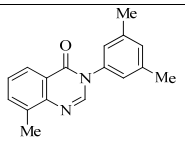
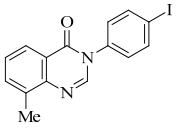
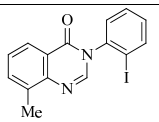
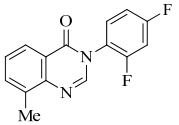
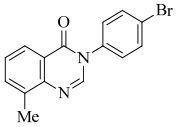
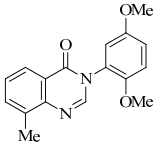
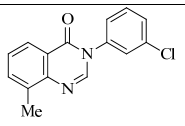
CODE	STRUCTURE	IUPAC Name	Molecular Formula	Molecular Weight
Q1		3-(4-chlorophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}ClN_2O$	270.71
Q2		3-(3-fluorophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}FN_2O$	254.26
Q3		3-(3-bromophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}BrN_2O$	315.16
Q4		8-methyl-3-(3-(methylthio)phenyl)quinazolin-4(3H)-one	$C_{16}H_{14}N_2OS$	282.36
Q5		3-(4-fluorophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}FN_2O$	254.26
Q6		3-(3,5-dimethylphenyl)-8-methylquinazolin-4(3H)-one	$C_{17}H_{16}N_2O$	264.32
Q7		3-(4-iodophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}IN_2O$	362.17
Q8		3-(2-iodophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}IN_2O$	362.17
Q9		3-(2,4-difluorophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{10}F_2N_2O$	272.25
Q10		3-(4-bromophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}BrN_2O$	315.16
Q11		3-(2,5-dimethoxyphenyl)-8-methylquinazolin-4(3H)-one	$C_{17}H_{16}N_2O_3$	296.32
Q12		3-(3-chlorophenyl)-8-methylquinazolin-4(3H)-one	$C_{15}H_{11}ClN_2O$	270.71

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Table 1. continued

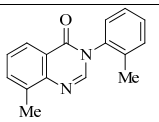
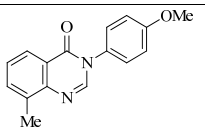
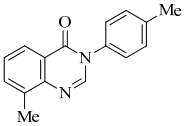
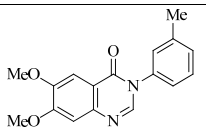
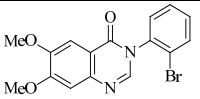
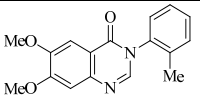
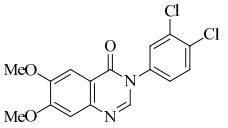
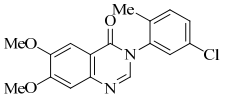
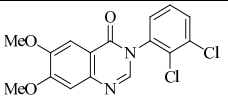
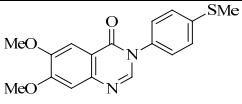
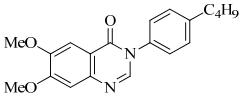
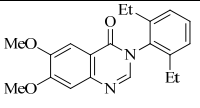
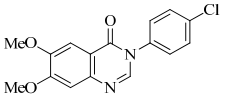
CODE	STRUCTURE	IUPAC Name	Molecular Formula	Molecular Weight
Q13		8-methyl-3-(o-tolyl)quinazolin-4(3H)-one	$C_{16}H_{14}N_2O$	250.30
Q14		3-(4-methoxyphenyl)-8-methylquinazolin-4(3H)-one	$C_{16}H_{14}N_2O_2$	266.29
Q15		8-methyl-3-(p-tolyl)quinazolin-4(3H)-one	$C_{16}H_{14}N_2O$	250.30
Q16		6,7-dimethoxy-3-(m-tolyl)quinazolin-4(3H)-one	$C_{17}H_{16}N_2O_3$	296.32
Q17		3-(2-bromophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{13}BrN_2O_3$	361.19
Q18		6,7-dimethoxy-3-(o-tolyl)quinazolin-4(3H)-one	$C_{17}H_{16}N_2O_3$	296.32
Q19		3-(3,4-dichlorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{12}Cl_{2N}2O_3$	351.18
Q20		3-(5-chloro-2-methylphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{17}H_{15}ClN_2O_3$	330.77
Q21		3-(2,3-dichlorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{12}Cl_2N_2O_3$	351.18
Q22		6,7-dimethoxy-3-(4-(methylthio)phenyl)quinazolin-4(3H)-one	$C_{17}H_{16}N_2O_3S$	328.39
Q23		3-(4-butylphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{20}H_{22}N_2O_3$	338.40
Q24		3-(2,6-diethylphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{20}H_{22}N_2O_3$	338.40
Q25		3-(4-chlorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{13}ClN_2O_3$	316.74

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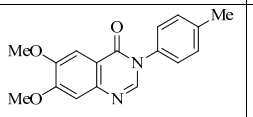
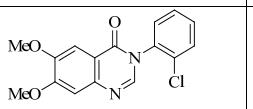
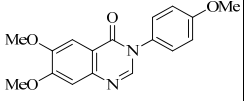
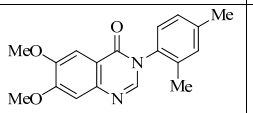
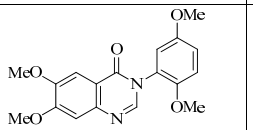
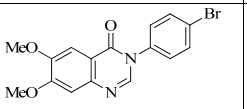
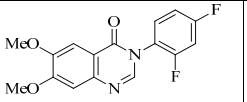
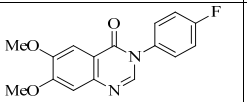
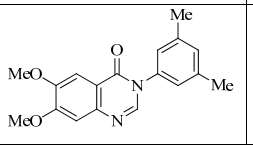
Table 1. continued

CODE	STRUCTURE	IUPAC Name	Molecular Formula	Molecular Weight
Q26		6,7-dimethoxy-3-(p-tolyl)quinazolin-4(3H)-one	$C_{17}H_{16}N_2O_3$	296.32
Q27		3-(2-chlorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{13}ClN_2O_3$	316.74
Q28		6,7-dimethoxy-3-(4-methoxyphenyl)quinazolin-4(3H)-one	$C_{17}H_{16}N_2O_4$	312.32
Q29		3-(2,4-dimethylphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{18}H_{18}N_2O_3$	310.35
Q30		3-(2,5-dimethoxyphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{18}H_{18}N_2O_5$	342.35
Q31		3-(4-bromophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{13}BrN_2O_3$	361.19
Q32		3-(2,4-difluorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{12}F_2N_2O_3$	318.27
Q33		3-(4-fluorophenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{16}H_{13}FN_2O_3$	300.28
Q34		3-(3,5-dimethylphenyl)-6,7-dimethoxyquinazolin-4(3H)-one	$C_{18}H_{18}N_2O_3$	310.35

[a]Name, molecular formula, structure, molecular weight, and code name of the quinazolinones are shown.

quinazolinone derivatives are depicted in Table 1. The compounds were numbered Q1−Q34 based on their arrangement in Table 1. Stock concentrations of 10 mg/mL were prepared in methanol and stored at room temperature. Solvent controls contained the same volume of methanol as the volume used from the stock solutions.

Henrietta Lacks (HeLa) and HaCaT Cell Culture. HeLa and HaCaT cells were used as a food source for *N. fowleri* and *B. mandrillaris* cells and for determination of cytotoxicity. Human cervical adenocarcinoma cells (HeLa) (ATCC CCL-2) were obtained from American Type Culture Collection, while human keratinized skin cells (HaCaT) (CLS:300493) were acquired from CLS Cell Lines. Cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% minimum essential medium amino acids, 1% L-glutamine, and 1% antibiotics (supplemented RPMI-1640) at 37 °C and 5% CO_2. When fully confluent, the cells were detached from the flasks using trypsin, transferred to a tube, spun at 1258*g* for 5 min, resuspended in supplemented RPMI-1640, and transferred into tissue culture flasks or tissue culture plates.[35,36]

Culture of Amoebae. *N. fowleri* cells isolated from the cerebrospinal fluid of a patient (ATCC 30174) as well as *B. mandrillaris* cells from the brain of a mandrill baboon (ATCC 50209) were utilized in this study. Amoebae were cultured as previously described.[37,38] Briefly, the amoebae were cultured in RPMI-1640 medium supplemented with 1% antibiotics with HeLa cell monolayers were used as food source at 37 °C in a 5% CO_2 incubator.

Amoebicidal Assays. Amoebicidal assays were performed as previously described.[38] Briefly, 5×10^5 amoebae cells were incubated with 100 μg/mL of drugs and/or solvents (methanol) in 24-well plates in RPMI-1640. Amoebae were incubated with RPMI-1640 for negative control. For positive controls, antiamoebic drugs, amphotericin B and ketoconazole were utilized at 100 μg/mL. Plates were incubated at 37 °C and 5% CO_2 for 24 h. The viability of amoebae was assessed by counting live (unstained) cells, after addition of 0.1% Trypan blue to each well, using a hemocytometer. The data are representative of the mean ± standard error of several independent experiments performed in duplicate.

Cytopathogenicity Assays. Cytopathogenicity assays were performed as previously described.[38] Briefly, 5×10^5 amoebae cells were treated with drugs for 2 h. Amoebae were then collected by centrifugation at 1258*g* for 5 min. The pellet was resuspended in RPMI-1640. The amoebae were incubated in RPMI-1640 in 24-well

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plates containing HeLa monolayers at 37 °C and 5% CO_2 and were observed until 50% damage was noted for untreated amoebae. The supernatant was then collected and tested for the presence of lactate dehydrogenase (LDH) enzyme using a cytotoxicity detection kit (Roche Diagnostics). The percentage LDH release was determined as follows: [(absorbance of sample − absorbance of control)/(absorbance for total LDH release − absorbance of control)] × 100 = percentage cytotoxicity. The data are representative of the mean ± standard error of several independent experiments performed in duplicate.

Amoebistatic Assays. For amoebistatic assays, 5 ×10^5 amoebae were incubated with 100 μg/mL drugs and/or solvent (methanol) in 24-well plates containing RPMI-1640 on HeLa monolayers at 37 °C and 5% CO_2 for 24 h. RPMI-1640 alone was used as a negative control, while 100 μg/mL amphotericin B and ketoconazole were used as positive controls. Live amoebae were then quantified as described for amoebicidal assay using Trypan blue exclusion assay. The data are representative of the mean ± standard error of several independent experiments performed in duplicate.

Scanning Electron Microscopy. Amoebae were fixed with 2.5% glutaraldehyde for scanning electron microscopy.[39] Amoebae were treated with drugs for 2 h and washed with phosphate buffered saline (PBS), fixed in 2.5% glutaraldehyde for 2 h, and resuspended in 50 μL of Milli-Q water. A volume of 10 μL of the suspension was then placed on a coverslip and left for 10 min to allow adherence and were dried overnight. Field-emission scanning electron microscopy (FE-SEM) (Hitachi SU8010) was then conducted to capture high magnification images of the treated and untreated amoebae.

Cytotoxicity Assays. Cytotoxic effects of the drugs were determined as previously described.[35,39] The cytotoxic activity of the drugs was investigated against HaCaT cells. Drugs at 100 μg/mL were incubated with HaCaT cells in RPMI-1640 for 24 h at 37 °C and 5% CO_2. The RPMI-1640 was then collected and tested for the presence of lactate dehydrogenase (LDH) enzyme by using a cytotoxicity detection kit (Roche Applied Science). The percentage LDH released was determined as follows: [(absorbance of sample − absorbance of control)/(absorbance for total LDH release − absorbance of control)] × 100 = percentage cytotoxicity.

Nanoparticle Conjugation. Silver nanoparticles (AgNPs) were conjugated with selected representative aryl quinazolinones by one phase reduction method as previously described.[38] Briefly, 1 mM quinazolinone was magnetically stirred with 1 mM silver nitrate aqueous solution, followed by addition of sodium borohydride aqueous solution as reducing agent. Addition of sodium borohydride caused a change in the color of the solution to yellow-brown, indicating formation of AgNPs. To maximize yield, the mixture was further stirred for 1 h. The procedure was repeated by varying volume ratios of aryl quinazolinones to silver nitrate solution, while still using sodium borohydride as a reducing agent. The same procedure was used to obtain unconjugated AgNPs for use in biological assays.

Characterization of Nanoparticle Conjugation Using UV−Vis Spectroscopy and Dynamic Light Scattering. UV−vis absorption spectra were used for measuring the characteristic surface plasmon resonance band of AgNPs as described previously.[40] Dynamic light scattering technique was used at 25 °C, and an angle of 90° to determine the size distribution and zeta potential of AgNPs particles as previously described.[40]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.9b00596.

UV−vis spectra, particle sizes, and zeta potentials of quinazolinones conjugated with silver nanoparticles (PDF)

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Author Contributions

M.R.M. and M.S.S. contributed equally. N.A.K. and R.S. established the idea and supervised the study. M.S.S. and S.M.S. synthesized molecules and characterized nanoparticles under the supervision of K.M.K. AA analyzed the data related to the synthesis and characterization. MRM conducted all the bioassays and prepared the first draft of manuscript. N.K. and R.S. corrected and finalized the manuscript for submission. The manuscript was submitted with the endorsement of all authors.

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Notes

The authors declare no competing financial interest.

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