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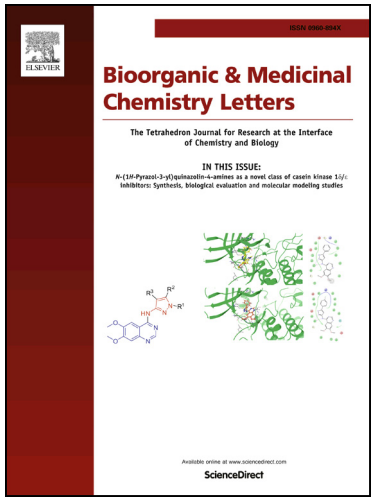

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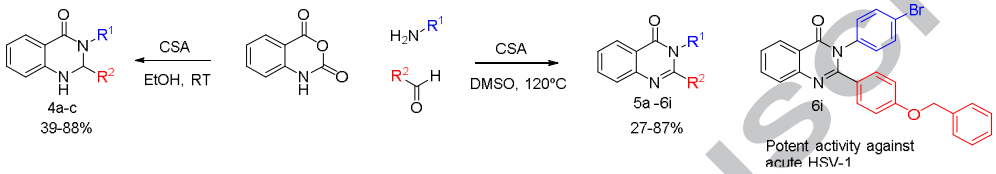

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Discovery of potent antiviral (HSV-1) quinazolinones and initial structure-activity relationship studies

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ABSTRACT

The discovery of antiviral activity of 2,3-disubstituted quinazolinones, prepared by a one-pot, three-component condensation of isatoic anhydride with amines and aldehydes, against Herpes Simplex Virus (HSV)-1 is reported. Sequential iterative synthesis/antiviral assessment allowed structure-activity relationship (SAR) generation revealing synergistic structural features required for potent anti-HSV-1 activity. The most potent derivatives show greater efficacy than acyclovir against acute HSV-1 infections in neurons and minimal toxicity to the host.

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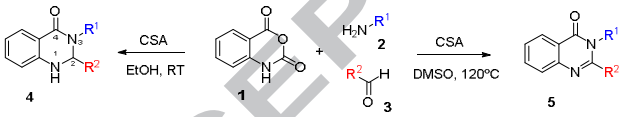
Herpes Simplex Virus 1 (HSV-1), a virus belonging to the family herpesviridae and a common human pathogen,[1] affects approximately 67% of the global population and is causing increasing concern for neonatal and immunocompromised patients.[2] HSV-1 displays both lytic and latent forms of infection in humans.[3] Recently, HSV-1 infection has been associated with cognitive impairment among persons with schizophrenia[4,5] and even persons without psychiatric illnesses.[6,7] Lytic HSV-1 infections are characterized by active viral replication causing substantial morbidity through recurrent cold sores, gingivostomatitis, and corneal infections that can culminate in blindness through stromal keratitis. While HSV-1 encephalitis is rare, the incidence of neonatal encephalitis is rising and survivors suffer from severe cognitive deficits. In the latency phase, the viral genome persists in neurons, only a handful of loci are transcribed. Latent infections can be reactivated by systemic or localized stress and is uncontrollable and unpredictable.[8] During latency, viral gene repression is controlled by host-cell epigenetic regulation, specifically by binding of deactylated histones to viral DNA, and reactivation of latent infections involves histone acetylation.[9,10] Reactivation of latent infections can be induced by treatment of histone deacetylase inhibitors.[11,12] HSV-1 is currently treated with nucleoside analogs such as acyclovir (ACV), but these drugs do not affect latent infection.[1] Additionally, resistance to ACV is increasing, particularly in immunocompromised patients who receive treatment for extended periods. There is a need to discover structurally novel compounds (i.e. non-nucleoside based) and/or mechanisms for treating lytic HSV-1 and reactivation.[13]

The quinazolinone core is found within both natural products and in pharmaceuticals, representing a privileged scaffold given the range of activity (antimicrobial,[14] antitubercular,[15] and anticancer activity[16]) noted for certain constituents. A number of quinazolinones have been identified as antiviral agents with activity against influenza,[17] HIV,[18] and TMV.[19] Quinazolinone analogs are also known to affect epigenetic regulation through inhibition of bromodomains (BET).[20] On the basis of these data, we hypothesized that quinazolinones may exhibit novel activity against HSV-1 and recently reported a comparative HSV-1 drug screening assay that demonstrated preliminary antiviral activity of



Scheme 1. The dichotomous solvent and temperature-dependent Bronsted acid catalyzed three component coupling of isatoic anhydride **1**, amines **2** and aldehydes **3** to yield dihydroquinazolinones **4** and quinazolinones **5**.

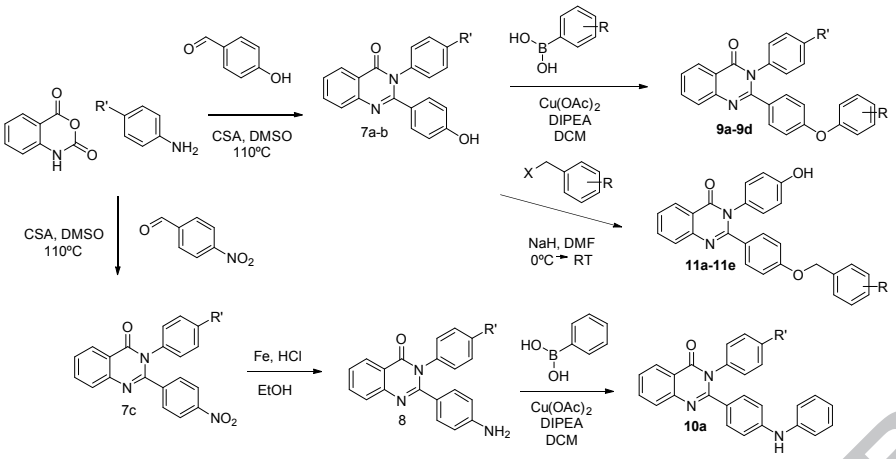
quinazolinones.[21] In this Letter, we describe the synthesis of dihydroquinazolinones **4** and quinazolinones **5** via a three-component coupling of isatoic anhydride **1**, amines **2** and aldehydes **3** (Scheme 1), optimizing this new potent antiviral HSV-1 pharmacophore through successive rounds of SAR.[21]

While the synthesis of quinazolinones has been reported through various methods, we were attracted to reports describing the condensation of *o*-aminobenzamides and carbonyl compounds.[22-30] These have been conducted under a variety of conditions including the use of Bronstead acid,[22,23] Lewis acid,[24] and other catalysts.[25,26] Aldehydes and ketones are common substrates, however diketones,[27] ß-ketoesters,[22] and alcohols[28] have also been incorporated. These reactions often require the prior preparation of the substituted *o*-aminobenzamide, thus involving at least two synthetic steps. In addition, to generate quinazolinones **5** from dihydroquinazolinones **4**, additional oxidants are sometimes employed.[28,29] Multicomponent approaches to quinazolinone synthesis have also been reported in the literature.[30] We initiated the present work directed toward development of a one-pot, three-component-coupling to quinazolinones (Scheme 1) following a recent report of such a process from isatoic anhydride, primary amines and aldehydes using catalytic iodine.[24] In our hands, following this procedure, the direct condensation of isatoic anhydride **1** with an amine **2** and aldehyde **3** was found to proceed readily in alcoholic solvents such as EtOH, however we found that incomplete oxidation yielded intractable mixtures of dihydroquinazolinone **4** and quinazolinone **5** products. A catalyst-free method employing urea and thiourea as ammonia equivalents has also been reported,[31] however under these conditions, no cyclization products were obtained.

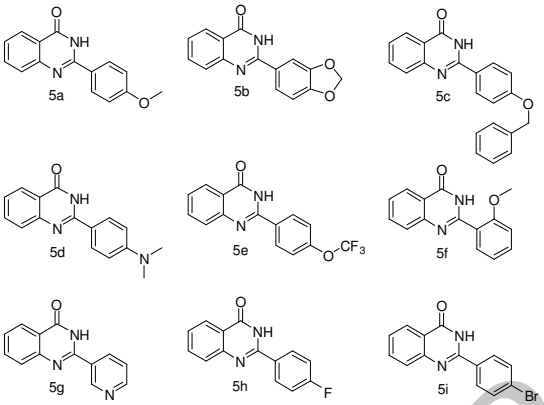
Further experimentation with various catalysts, solvents and temperatures resulted in the discovery of distinct reaction conditions leading exclusively to either dihydroquinazolinone **4** or quinazolinone product **5**, separately through one-pot processes. The reaction of isatoic anhydride, NH_4OAc or an amine, and aldehydes or ketones using a catalytic amount of the mild Bronsted acid camphor sulfonic acid (CSA) (Scheme 1) conducted in ethanol at room temperature led to dihydroquinazolinone **4** products in 39-88% isolated yield. In all cases, the product precipitated from the reaction mixture and could be purified simply by washing with ethanol. The dihydroquinazolinones proved to be devoid of antiviral activity in all cases and were not further pursued in the present investigation.

In contrast, when the same reaction was performed in dimethyl sulfoxide (DMSO) at 110 ˚C in air with a catalytic amount of CSA, aromatic quinazolinone products **5** were isolated exclusively. The 2-substituted quinazolinones so prepared precipitated as described for the dihydroquinazolinones leading to a first generation of derivatives **5a**-**5i**, Figure 1 (top). A second generation of 2,3-disubstituted quinazolinones **6a**-**6i**, Figure 1 (centre) was similarly accessed from anilines or amines in isolated yields of 27-83%.

The array of compounds (Figure 1) was screened for activity against acute HSV-1 using a previously published protocol.[32] Briefly, iPSC-derived neurons were infected with recombinant HSV-1 expressing EGFP under the control of the ICP0 promoter. iPSC-neurons were then treated with quinazolinones two hours after infection. After 72h, the number of fluorescent cells was measured by flow cytometry (See Supplementary Figure S1). ACV was used as a positive control, and the % fluorescent cells was normalized to the infected, untreated control. At 50 µM, **6c** and **6i** showed decreased % fluorescent cells compared to untreated controls (Figure 1). This indicates reduced expression of ICP0 and thus inhibition of viral replication. The structure-activity



Scheme 2. Synthesis of 2nd generation quinazolinones. Phenols **7a-b** can be coupled with aryl boronic acids or aryl halides to afford diaryl ethers and benzylethers. Aniline **8** was used to prepare the diarylamine analog **10**.



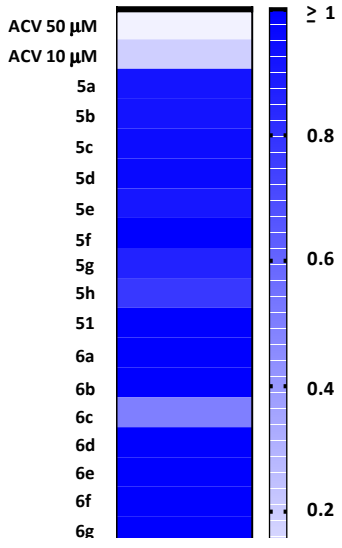
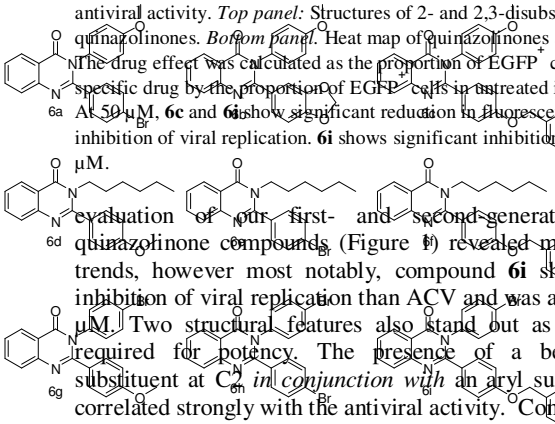


Figure 1. Analysis of 2- and 2,3-disubstituted quinazolinones and their antiviral activity. *Top panel:* Structures of 2- and 2,3-disubstituted quinazolinones. *Bottom panel*. Heat map of quinazolinones antiviral activity. The drug effect was calculated as the proportion of EGFP+ cells exposed to a specific drug by the proportion of EGFP+ cells in untreated infected cultures. At 50 µM, **6c** and **6i** show significant reduction in fluorescence, indicating inhibition of viral replication. **6i** shows significant inhibition of HSV-1 at 10 µM.



evaluation of our first- and second-generation series of quinazolinone compounds (Figure 1) revealed many interesting trends, however most notably, compound **6i** showed stronger inhibition of viral replication than ACV and was also active at 10 µM. Two structural features also stand out as synergistically required for potency. The presence of a benzyloxybenzyl substituent at C2 *in conjunction with* an aryl substituent at N3 correlated strongly with the antiviral activity. Compounds **5c** and **6f** containing a C2 benzyloxybenzyl substituent and an alkyl or no substituent at N3 proved relatively inactive. The activity of the series **6g**, **6h** and **6i,** containing the same 4-bromophenyl substituent at N3, highlights the superior activity of the C2 benzyloxybenzyl substituent. Compound **6i**, containing a 4-bromophenyl substituent at N3 was more active than **6c** and **5c**, which contain an electron rich 4-methoxyphenyl moiety or hydrogen at N3. The overall results demonstrate that quinazolinones with a benzyloxybenzyl group at C2 and an electron deficient 4-bromophenyl group at N3 work synergistically in this new potent anti-HSV-1 pharmacophore.

Compounds that showed activity in our initial screen were further tested for toxicity against iPSC-neurons using a LIVE/DEAD Fixable Aqua dead cell stain kit according to previously published protocols.[32] Although **6c** and **6i** had little affect on cell viability,[21] we observed some morphological

changes to neurons after they had been treated with **6c** and **6i** for 48-72 hours. As this toxicity was not immediately observed, we hypothesized that these molecules could be unstable under the assay conditions, and thus decomposing or being metabolized to more toxic species. We identified the benzyloxybenzyl group as a particularly labile site. The functional group is crucial for anti-HSV activity, but we thought analogs with similar large, hydrophobic groups at C2 might retain antiviral activity while minimizing toxicity to the host. We thus envisioned a third generation of quinazolinones with substituted benzyl ethers, diaryl ethers, and diaryl amines at C2.

To prepare the third generation of quinazolinones, compounds **7a-b** were prepared from the cyclization of isatoic anhydride, 4-hydroxybenzaldehyde, and p-anisidine (**7a**) or 4-bromoaniline (**7b**) (Scheme 2). These phenols were then coupled to boronic acids under Chan-Lam[33] conditions affording diaryl ethers, or alkylated using benzyl halides producing benzyloxybenzyl ethers. A number of diaryl ethers and benzyloxybenzyl ethers with different substituents on the terminal aryl ring were prepared. Additionally, a diarylamine analog was prepared from the three component coupling of isatoic anhydride, p-anisidine, and 4-nitrobenzaldehyde, followed by the reduction of the nitro group and Chan-Lam coupling. In total, a collection of ten third generation compounds was prepared, summarized in Figure 3.

Our third-generation quinazolinones were again tested for activity against HSV-1 (Figure 2). To our surprise, replacement of the benzyloxybenzyl substituent with a diaryl ether (**9a-d**) or a diaryl amine (**10a**) completely eliminated antiviral activity. This large difference in activity suggests the terminal ring in the C2 substituent is very important for anti-HSV activity. The benzyl methylene may be required for this ring to reach its binding site, either as a spacer or by providing additional flexibility to the chain. Some of the substituted benzyloxybenzyl ethers (**11a, 11c, 11d-e**) did retain activity against HSV-1. The derivatives containing a 3- or 4-bromobenzyloxybenzyl moiety were more active than the corresponding electron rich 4-methoxybenzyloxybenzyl analog.

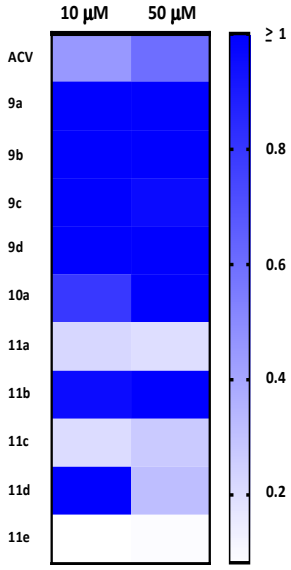


Figure 2. Analysis of third generation quinazolinones containing diaryl ether, benzyloxyether, and diaryl amine functionality. *Top panel:* Molecular structures. *Bottom panel:* Heat map of antiviral activity. The drug effect was calculated as the proportion of $EGFP^+$ cells exposed to a specific drug by the proportion of $EGFP^+$ cells in untreated infected cultures. At 10 µM and 50 µM, **11a**, **11c**, **11d**, and **11e** show significant reduction in fluorescence, indicating inhibition of viral replication.

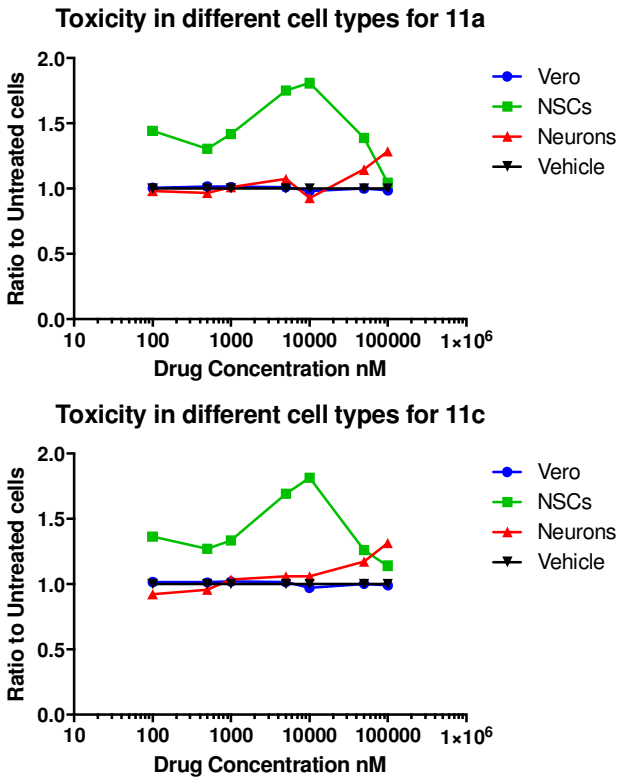


Figure 3. Toxicity of **11a** and **11c** to Vero cells, neural stem cells, and neurons compared to vehicle. No decrease in cell viability is observed at high concentrations, and at low concentrations a small increase in cell viability is apparent.

This may be due to the changes induced in the electronegativity of the benzyl ring. Additionally, we were pleased to find that **11a** and **11c** were less toxic than **6c** and **6i** (Figure 3). This suggests substitution of the benzyl ring may prevent oxidation of this compound by CYP450 enzymes and thus minimize the formation of toxic biproducts.

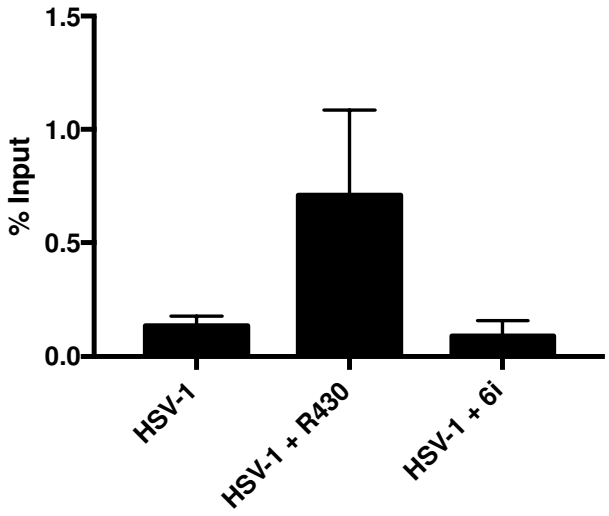


Figure 4: Enrichment of triMe H3K27 at ICP4 gene in acutely infected iPSC-derived neurons exposed to **6i**. ChIPs using anti-triMe H3K27 were subjected to real-time PCR using primers specific for HSV-1 ICP4 promoter, and the results were graphed as % input normalized to RHO %Input. In contrast to R430 (trans-dihydrolycoricidine)[13] **6i** does not induce heterochromatinization.

Given that these compounds are potent inhibitors of acute HSV-1, we next determined their ability to induce quiescence. A chromatin immunoprecipitation (ChIP) assay was performed to study the effect of **6i,** the most potent derivative, on heterochromatin dimerization. In this assay, cells were infected with HSV-1 and treated with **6i** or R430, a drug we have proposed to alter HSV-1 latency.[13] This was followed by harvesting, fragmentation of chromatin, and analysis of the enrichment of H3K27Me3 at the viral ICP4 promoter. H3K27Me3 was then normalized to rhodopsin (RHO). Compared to acutely infected cells, cells treated with **6i** showed no enrichment of H3K27Me3, indicating that the compound does not induce heterochromatization (Figure 4) indicating that **6i** is active against acute HSV-1 infection and does not induce quiescence.

In conclusion, the hypothesis of potential antiviral activity of quinazolinones indeed led to the discovery of quinazolinones with potent activity against acute HSV-1. A clean, dichotomous process was developed to prepare dihydroquinazolinones or quinazolinones selectively. Through three successive rounds of SAR, we determined that the most potent compounds against HSV-1 synergistically constitute aryl substitution at N3 and a benzyloxybenzyl moiety at C2. This pharmacophore appears to be quite specific, modifying either C2 or N3 substituents eliminates activity. These quinazolinones show little host-cell cytotoxicity and do not induce heterochromatization in HSV-1 infected cells, indicating activity against replicating HSV-1. Nonetheless, the quinazolinones are structurally distinct from nucleoside-based therapeutics such as ACV and thus represent a new class of experimental antiviral agent. Further development of this new pharmacophore, investigation against other viral pathogens and studies on the mechanism are under active investigation in our laboratories.

Acknowledgments

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