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An integrated chemical biology approach reveals the mechanism of action of HIV replication inhibitors

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ABSTRACT

Continuous flow (microfluidic) chemistry was employed to prepare a small focused library of dihydropyrimidinone (DHPM) derivatives. Compounds in this class have been reported to exhibit activity against the human immunodeficiency virus (HIV), but their molecular target had not been identified. We tested the initial set of DHPMs in phenotypic assays providing a hit (**1i**) that inhibited the replication of the human immunodeficiency virus HIV in cells. Flow chemistry-driven optimization of **1i** led to the identification of HIV replication inhibitors such as **1l** with cellular potency comparable with the clinical drug nevirapine (NVP). Mechanism of action (MOA) studies using cellular and biochemical assays coupled with 3D fingerprinting and *in silico* modeling demonstrated that these drug-like probe compounds exert their effects by inhibiting the viral reverse transcriptase polymerase (RT). This led to the design and synthesis of the novel DHPM **1at** that inhibits the replication of drug resistant strains of HIV. Our work demonstrates that combining flow chemistry-driven analogue refinement with phenotypic assays, *in silico* modeling and MOA studies is a highly effective strategy for hit-to-lead optimization applicable to the discovery of future therapeutic agents.

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1. Introduction

In broad terms the process of drug discovery can be separated into two different but well-defined approaches. The target-based approach consists of identifying a suitable protein target and screening chemical libraries of small molecules against this target to identify hits that may be optimized into drugs. The phenotypic drug discovery approach involves screening in cells or organisms where the target is unknown and the mechanism of action (MOA) of active compounds is deduced later through a process of deconvolution. More recently the use of targeted compound libraries based on so-called "privileged" scaffolds that are known to have biological activity against one target class have been used to screen against other targets to identify hits.[2–9] All of these approaches are either target-based or phenotype-based and originate in searches for small molecule therapeutic agents for a specific disease, as discussed in detail by Schenone and colleagues.[6] We have refined a different approach. Our compound-centric methodology that starts with highly promising scaffolds, identifies a putative therapeutic area where these scaffolds may prove beneficial, and subsequently identifies a molecular target for the compound action. We have exemplified this novel strategy to generate new classes of bio-active compounds based on our ability to rapidly generate diverse dihydropyrimidinone (DHPM) derivatives.[10]

DHPMs are well known as privileged scaffolds that exhibit favorable therapeutic and pharmacological properties across a range of bioactivities in approved drugs.[11,12] A comprehensive analysis of published bioactivities of DHPMs with some similarity to our derivatives led us to investigate activity against human immunodeficiency virus (HIV),[13] a therapeutic target that is amenable to phenotypic screening. HIV is the causative agent of acquired immunodeficiency syndrome (AIDS), a disease for which there is no cure. Currently, more than 1.1 million people in the USA are living with HIV infection. The prevalence of HIV/AIDS is on the rise with about 50,000 new infections each year in the USA alone. The cost of these new cases is estimated to be around $36.4 billion, comprised of both direct medical costs ($6.7 billion) and loss of productivity ($29.7 billion).[14] A comprehensive analysis of the contemporary cost of HIV healthcare by Gebo and colleagues

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concluded: "*HIV healthcare in the United States continues to be expensive, with the majority of expenditures attributable to medications. With improved HIV survival, costs may increase and should be monitored in the future.*"[15] Despite the fact that there are over 25 FDA approved drugs for the treatment of HIV,[16] the well-documented ability of the virus to acquire resistance against established methods of treatment requires the development of novel drug classes. There is therefore an ongoing significant need to investigate probe molecules that could ultimately lead to drugs with the capacity to overcome such resistance.[17,18]

Herein we describe how screening a focused library of small molecule compounds in a phenotypic assay coupled with flow chemistry-driven hit-to-lead optimization, comprehensive database searching and computational modeling led to the prediction and subsequent identification of the MOA for a novel series of HIV replication inhibitors. Our efficient approach to target identification and validation may provide the basis for an expanded drug discovery program.

2. Results and discussion

We recently reported a highly efficient continuous flow method for the synthesis of substituted dihydropyrimidinone (DHPM) derivatives (**1**).[10] Our methodology provides access to a scaffold with favorable drug-like characteristics and allows the generation of highly varied analogues. The compounds prepared in this way are structurally related to thiazole derivatives such as compound **2** that were initially disclosed in a patent application and subsequently reported in the scientific literature (Fig. 1).[13] We were intrigued by the physical similarity between our DHPM derivatives and the related structures **2**, which were described to possess inhibitory activity toward HIV replication in cells, although no MOA has been reported.[13] We therefore designed a biological testing funnel to narrow down the target of the DHPMs (Fig. 2). We initiated target identification studies by comparing antiviral activity in two commonly used phenotypic screens. The MAGI-CCR5 antiviral assay is a single round antiviral assay employing the MAGI-CCR5 reporter cell line that produces low levels of virus. In this assay, the virus produced in an infected cell does not infect neighboring cells. MAGI-CCR5 cells can therefore be used to assess the MOA of compounds that are active early in the replication cycle, such as entry or viral reverse transcriptase polymerase (RT) inhibitors. We utilized peripheral blood mononuclear cells (PBMC) in a multi-round assay as the comparator assay.[19] In contrast to a single-round assay, in the PBMC assay the neighboring cells are infected by the progeny virions produced from the initial infection. Using control compounds as a reference, a difference of greater than two-fold activity was required in order to convince us that the activity was localized to early steps in the replication cycle. The result of this dual assay approach allowed for the confirmation of antiviral activity while already narrowing the MOA. We tested an initial set of analogues in cellular antiviral assays measuring HIV replication alongside cytotoxicity prior to advancing to more in-depth and costly secondary studies to identify the MOA. The data generated from the first set of compounds prompted us to investigate the SAR in more detail utilizing the already established phenotypic assay and led to a new series of potent HIV replication inhibitors with drug-like properties. The most promising compound was analyzed relative to known HIV inhibitors and a hypothesis was generated that the DHPM target was the HIV RT. Molecular modeling studies along with target specific biological assays confirmed this hypothesis. Our approach to the discovery, rapid SAR and characterization of a series of new HIV replication inhibitors is described in detail below.

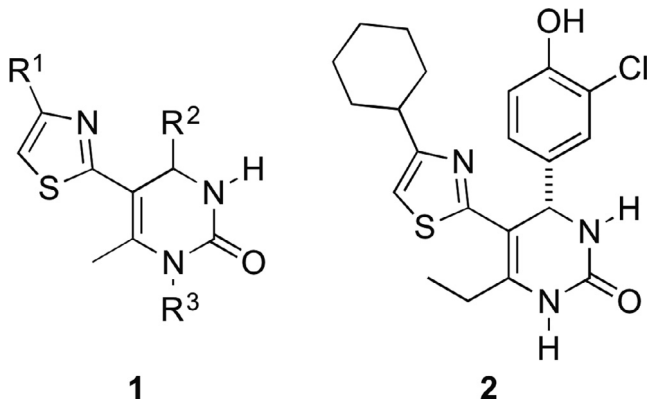

Fig. 1. 5-(Thiazol-2-yl)-3,4-dihydropyrimidin-2(*1H*)-one derivatives.

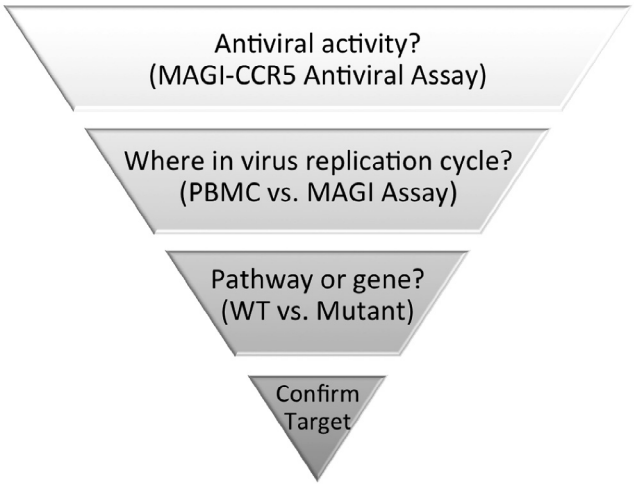

Fig. 2. Testing funnel summarizing the biological assays employed in our study.

2.1. Library construction

The multistep microfluidic synthesis of DHPM analogues **1** is shown in Scheme 1.[10] At the outset of our experiments, we elected to first vary the α-bromoketones **4** to investigate the effects of changing the thiazole R^1 substituent while keeping the DHPM R^2 substituent constant (*i.e.* R^2 = 3-hydroxyphenyl). Thus, 0.75 M solutions of thioamide **3** and α-bromoketones **4** in DMF were pumped (32.5 μL/min) into a 250 μL reactor heated to 150 °C for 3.75 min. The stream exiting the first microreactor containing each newly constructed ketothiazole intermediate **7** was introduced to a separate stream (32.5 μL/min) of 3-hydroxybenzaldehyde **5a** and urea (**6a**, R^3 = H) (0.90 M, DMF). The combined flow of reactants (97.5 μL/min) was pumped into a 1000 μL reactor heated at 200 °C to generate new DHPM derivatives (**1a**–**1k**). Overall, the continuous two-chip microfluidic sequence required less than one hour for completion from start to finish (injection, reaction, and 1250 μL collection). The yields for the three-step (thiazole formation/deprotection/Biginelli reaction), two-chip sequence were high (39–48%) (Table 1) and provided sufficient quantities of compounds **1a**-**1k** for characterization and evaluation in cellular antiviral assays (Table 1). Of the eleven entries, only the extremely electron poor α-bromoketone **4e** (4-trifluoromethylphenyl) failed to furnish product using this methodology and thus **1e** was prepared using a one-pot batch procedure using conditions optimized using our flow method (Scheme 2). It is notable that the reaction

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