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Mini-review

Assembling a smallpox biodefense by interrogating 5-substituted pyrimidine nucleoside chemical space

Xuesen Fan [a], Xinying Zhang [a], Longhu Zhou [a], Kathy A. Keith [b], Earl R. Kern [b], Paul F. Torrence [a,*]

[a] *Department of Chemistry and Biochemistry, Northern Arizona University, Flagstaff, AZ 86011, USA*
[b] *Department of Pediatrics, University of Alabama School of Medicine, Birmingham, AL 35233, USA*

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven, in September 2006.

Abstract

The nucleoside 5-formyl-2′-deoxyuridine has been used as a starting point for the generation of novel 5-substituted pyrimidine nucleosides that are shown to possess significant antiviral activity against two representative orthopoxviruses, namely vaccinia virus and cowpox virus.

Keywords: Cowpox virus; Vaccinia virus; Aldehyde; Pyran; Pyrazolone; Multicomponent reaction

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1. Introduction

Smallpox is one of the most devastating of infectious diseases (Esposito and Fenner, 2001) with a fatality rate of 30% or more (Torrence, 2005). In the 20th century alone, smallpox deaths worldwide numbered in the millions. Smallpox has killed more humans than all the wars of all time (Abrahams and Kaufman, 2004; Ales and Katial, 2004; Balicer et al., 2005; Bossi et al., 2005; Bray, 2005; Bricaire and Bossi, 2004; Cassimatis et al., 2004; Chen, 2004; Eckart et al., 2004; Ein et al., 2005; Guharoy et al., 2004; Harrop et al., 2004; Torrence, 2005). It is readily spread person-to-person by aerosol in any climate or season.

In 1983, the World Health Organization (WHO) declared smallpox eradicated as a natural disease. By 1983, all known stocks of variola virus were destroyed except for those in two WHO collaborating centers: the US Centers for Disease Control and Prevention (CDC) in Atlanta and (after a transfer in 1994) the Russian State Research Center of Virology and Biotechnology (the Vektor Institute) in Novosibirsk. The WHO Committee on Orthopoxvirus Infections voted on several occasions to recommend destruction of all virus stocks, but each time the decision was deferred to permit more research on live variola virus.

The concern that undeclared stocks of variola virus might exist and that they might be used as a bioterrorist weapon was heightened in late 2001 by the deliberate release of *Bacillus anthracis*, the agent of anthrax, in the weeks after the September 11, 2001 attacks. Further concern has arisen from the remote possibility of clandestine development of a genetically engineered more virulent variola strain. Smallpox vaccinations stopped in the US in 1972; moreover, vaccination immunity acquired before that time may not be protective.

* Corresponding author. Tel.: +1 928 523 3008; fax: +1 928 523 8111.
E-mail address: Paul.Torrence@nau.edu (P.F. Torrence).

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Medical treatment of smallpox is limited to supportive therapy and antibiotics as required for treating secondary bacterial infections. Vaccination is effective against smallpox only if given before exposure or no later than 2–3 days after exposure. One drug, cidofovir (Vistide®), is licensed to treat cytomegalovirus (CMV) retinitis in HIV-infected patients and cidofovir is available through a special protocol (Investigational New Drug, IND) for emergency treatment of smallpox or vaccine reactions (http://www.bt.cdc.gov/agent/smallpox/vaccination/cidofovir.asp) if vaccine immune globulin (VIG) does not work. Cidofovir might also be used to treat generalized vaccinia, eczema vaccinatum or progressive vaccinia. Some individual agents for the treatment of orthopoxvirus infections are in preclinical or clinical development. These include inhibitors of viral morphogenesis (TTP-6171) (Byrd et al., 2004) and viral release (ST-246) (Yang et al., 2005a) as well as cellular, i.e. Erb-1 kinase inhibitors (CI-1033) (Yang et al., 2005b; Fauci and Challberg, 2005) and tyrosine kinase inhibitors (Gleveec, STI-571) (Reeves et al., 2005).

Ultimately, the development of antiviral drugs against smallpox could deter rogue states and terrorists from releasing variola virus because its impact would be diminished. Very importantly, a non-toxic orally active efficacious antiviral against orthopoxviruses could be used prophylactically in unvaccinated populations until vaccinations had sufficient time to "take", and could be the primary line of defense for those unable to be vaccinated. Such prophylactic or therapeutic antivirals would serve as a strong secondary defense against the specter of an unusually virulent orthopoxvirus agent generated by selection or by genetic engineering. Several such antivirals, targeting different aspects of the virus replication apparatus, would act to cover such an Achilles heel in biodefense against orthopoxvirus bioterrorism or biowarfare. It is, in fact, the stated role of the US Government to have available two anti-smallpox drugs possessing different mechanisms of action and to have two more such drugs in the pipeline (Tseng, 2005). Such therapeutic agents would also be useful for the treatment of the emerging disease of monkeypox (Su, 2004; Torres-Velez and Brown, 2004).

2. Results and discussion

The foundation of our ongoing approach to orthopoxvirus antivirals may be divided into three premises.

I. The variola virus genome codes for approximately 200 gene products; however, only a handful of these are known. Although target-oriented drug discovery is an end devoutly to be desired and has been realized in the case of a recently described inhibitor of viral maturation (Byrd et al., 2004), it is unlikely there will be any full description of the variola virus proteome in the foreseeable future, although a proteomics database of the open reading frames of the Copenhagen strain of vaccinia virus is available (Randall et al., 2004). Nonetheless, this expressed proteome provides a plethora of potential prey to the alternative drug discovery approach driven by new chemistries. In this approach, small molecules are screened for their capacity to modulate a given biological process. Serendipity remains a player, but success can be made by a more frequent visitor to the table through diversity-oriented synthesis that is informed by rational design aligned with input from ongoing endpoint assays. This strategy not only can result in therapeutically useful entities, but it can also lead to the identification of relevant disease-related target macromolecules for further exploitation.

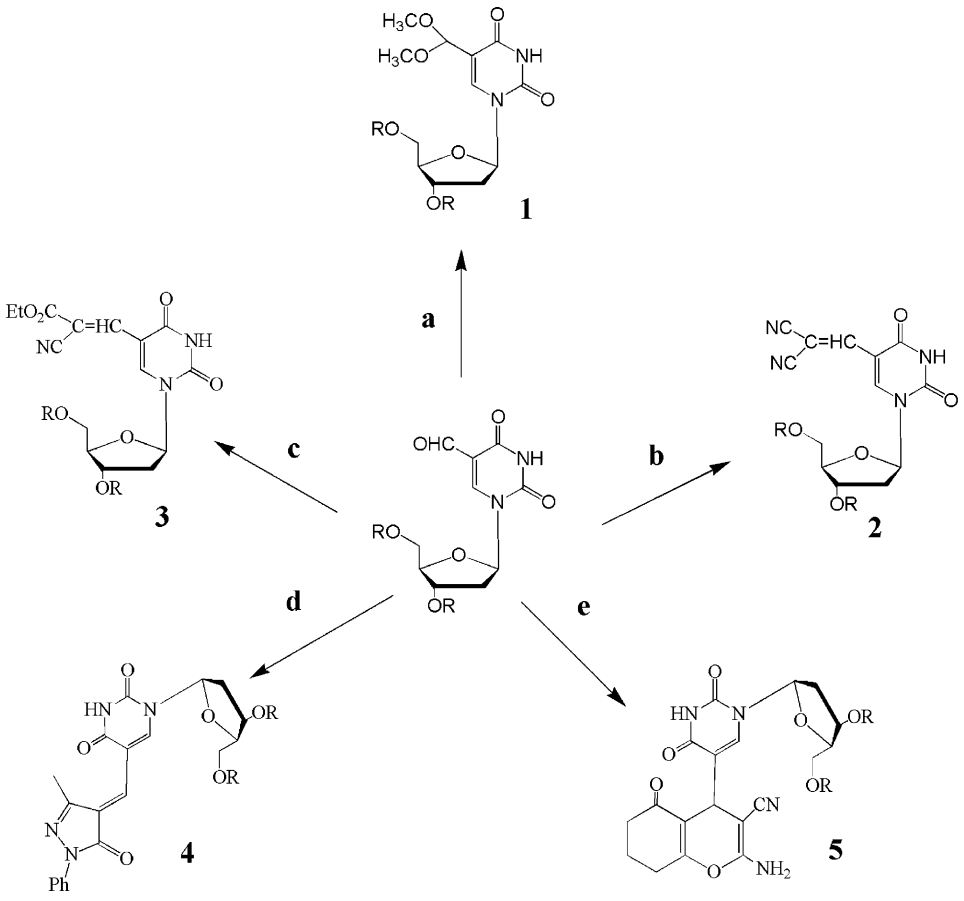
II. The "privileged" structure of nucleosides has led to a variety of efficacious antiviral agents such as 3′-azido-3′-deoxythymidine (zidovudine, AZT), stavudine (d4T), lamivudine (3TC), abacavir (ABC), didanosine, zalcitabine, acyclovir, ganciclovir, valaciclovir, cidofovir, tenofovir, ribavirin, brivudin, famciclovir, penciclovir, emtricitabine (FTC), adefovir and valganciclovir. Moreover, a number of 5-substituted pyrimidine nucleosides have quite potent anti-vaccinia virus activity. As reviewed by De Clercq (2001), these include 5-trifluoromethyl-, 5-nitro-, 5-formyl-, 5-ethynyl-, 5-amino- and 5-cyano-2′-deoxyuridines. These most probably have thymidylate synthase as their target. Thus, the nucleoside scaffold represents an excellent point of departure in the search for new antiviral drugs including one targeting orthopoxviruses. In addition, it is possible to generate chimeric molecules that incorporate the privileged scaffolds of other successful drugs and biologically active molecular classes.

III. Pyrimidine nucleosides as a class have demonstrated potent and selective antiviral activities against a range of viruses. To wit, consider the established therapeutics such as AZT, dideoxycytidine (ddC), lamivudine (3TC), 2′,3′-didehydro-3′-deoxythymidine (d4T) and BVDU (brivudin). In addition, other 5-substituted pyrimidine nucleosides have demonstrated impressive antiviral potency in cell culture including 5-iodo-, 5-trifluoromethyl-, 5-nitro-, 5-thiocyano- and 5-ethyl-2′-deoxyuridine, to name just a few (reviewed by De Clercq, 2001)

Our cornerstone for an unbiased exploration of 5-substituted pyrimidine nucleoside chemical space has been the known 5-formyl-2′-deoxyuridine which recruits the rich and extensive chemistry of the aldehyde carbonyl to this undertaking. We hypothesize that it will be possible to use this novel nucleoside diversomer library to discover and further develop at least one useful therapeutic and/or prophylactic agent for smallpox as well as other orthopoxvirus infections. As a corollary, we hypothesize that new targets for orthopoxvirus infection intervention will be uncovered. Finally, as a highly significant spin-off, leads will be discovered for the treatment of other viral diseases.

We have generated novel pyrimidine nucleoside analogues with a wide spectrum of structural diversity with attendant significant differences in such physicochemical variables as, but not limited to, polarity, electronic field effects, lipophilicity, steric bulk and hydrogen bond acceptor and donor potentials. The approach proffered here already has led to a variety of new nucleosides, several of which are described herein.

A significant advantage to this approach is the accessible synthetic chemistry that offers itself to analogue generations as



Scheme 1. Synthesis of nucleoside diversomers from 5-formyl-2′-deoxyuridine. Reagents and reaction conditions: (a) MeOH, amberlite IR-120 (H^+), reflux, 2 h; (b) malononitrile, EtOH, room temperature, overnight; (c) ethyl cyanoacetate, EtOH, room temperature, overnight; (d) 3-methyl-1-phenyl-2-pyrazolin-5-one, ethanol, room temperature, overnight; (e) malononitrile, 1,3-cyclohexanedione, ethanol, room temperature, overnight. R in Scheme 1 refers to either H or acetyl substituents.

well as the larger scale preparations that would be required for pharmacodynamics, toxicity evaluations and extension to various in vivo models of orthopoxvirus infections. Some of the relevant chemical metatheses are shown in Scheme 1. 5-Formyl-2′-deoxyuridine itself is readily obtained, in moderate yield, from thymidine. As an example, preparation of the dimethylacetals started with the known 3′,5′-di-*O*-acetyl-5-formyl-2′-deoxyuridine, which was prepared through the oxidation of 3′,5′-di-*O*-acetylthymidine with potassium peroxysulfate ($K_2S_2O_8$) in the presence of $CuSO_4$ and 2,6-lutidine in aqueous acetonitrile. 3′,5′-Di-*O*-acetyl-5-formyl-2′-deoxyuridine dimethyl acetal was prepared by refluxing the methanol solution of the aldehyde in the presence of an acidic resin used as a catalyst. This acetal was then transformed into its deacetylated counterpart, 5-formyl-2′-deoxyuridine dimethyl acetal by treatment with NH_3/MeOH at ambient temperature. Synthetic access to the dicyanovinyl congener was direct and afforded a high yield by reacting malononitrile with 5-formyl-2′-deoxyuridine or its diacetate at room temperature overnight. Likewise, ethyl cyanoacetate reacted at room temperature with 5-formyl-2′-deoxyuridine to provide the corresponding cyanovinyl carboxylate (Scheme 1).

Similarly (Scheme 1), the pyrazolone derivative was prepared through the condensation of an appropriate aldehyde with 1-phenyl-3-methyl-2-pyrazolin-5-one by stirring a solution of the nucleosidic aldehyde with one equivalent of 1-phenyl-3-methyl-2-pyrazolin-5-one in ethanol at room temperature for 6 h. The modified benzopyran derivative was generated by reaction among the 5-formyl-2′-deoxyuridine (or its diacetate), 1,3-cyclohexanedione and malononitrile.

All of these novel agents (Fig. 1 and Table 1) possessed in vitro antiviral activities that exceeded the activity of cidofovir against vaccinia and cowpox viruses in these in vitro

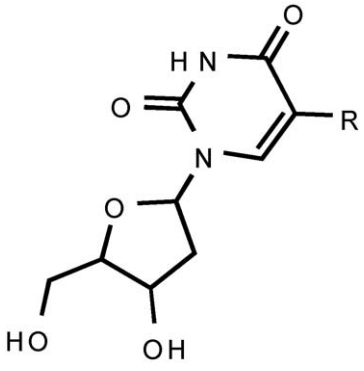
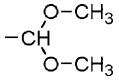
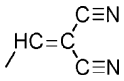
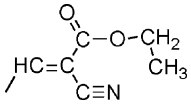
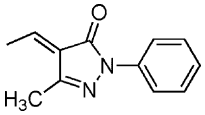
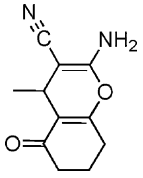


Fig. 1. 5-Substituted pyrimidine nucleosides.

Table 1
Anti-orthopoxvirus activities of novel nucleosides

Compound	Pyrimidine 5-substituent (R in Fig. 1)	Efficacy EC_{50}[a] (μM)				Toxicity CC_{50}[b] (μM)
		Vaccinia[c] CPE	Vaccinia[c] PR	Cowpox[c] CPE	Cowpox[c] PR	Neutral red uptake
Cidofovir	–	3.2	24 ± 12	7.1	40 ± 6.1	>317 ± 0
1		8.4	9.0 ± 1.3	11.7	7.4 ± 3.0	>300 ± 0
2		9.3	17 ± 6.4	<0.032	21 ± 12	>263 ± 64
3		10.7	18 ± 14	0.95	11 ± 9.2	>300 ± 0
4		1.7	6.9 ± 0.9	0.3	5.6 ± 5.2	>286 ± 25
5		0.6	4.6 ± 2.0	1.2	2.0 ± 0.3	>300 ± 0

Assays were performed according to the procedures described previously for activity against vaccinia virus (VV) and cowpox virus (CV) and for cytotoxicity (neutral red uptake assay) in human foreskin fibroblast (HFF) cells (Keith et al., 2003). Briefly, to determine efficacy, initial cytopathogenic effect (CPE) assays were performed in 96-well plates seeded with HFF cells. Varying concentrations of drug were challenged with VV or CV at 1000 PFU per well (incubation at 37 °C for 7 days). Subsequent confirmatory assays involving plaque reduction (PR) were performed using HFF cells seeded in six-well plates 2 days prior to use and infected with either VV or CV by the addition of 20–30 PFU per well. Plates were incubated for 1 h, various concentrations of drug were then added to triplicate wells and plates were incubated at 37 °C for 3 days. Toxicity was evaluated using HFF cells seeded in 96-well plates incubated with various concentrations of drug for 7 days at 37 °C.

[a] EC_{50}, effective concentration that reduced viral cytopathogenicity or plaque formation by 50%.

[b] CC_{50}, concentration which causes a cytotoxic effect (as ascertained by neutral red uptake) on 50% of uninfected cells.

[c] Virus used for challenge: vaccinia virus (Copenhagen) or cowpox virus (Brighton).

studies. The most active compounds were 5-(dimethoxymethyl)-2′-deoxyuridine (**1**), the pyrans (**5**) and the pyrazolone (**4**). The dicyanovinyl congener (**2**) and the cyano-carboethoxyvinyl congener (**3**) were of intermediate activity. The anomalous cowpox virus CPE-derived EC_{50} of the dicyanovinyl analogue (**2**) is noteworthy. There were some minor differences in the relative antiviral activity of these agents against vaccinia virus, on the one hand, and against cowpox virus on the other hand. Using the ratio of cowpox EC_{50}/vaccinia EC_{50} (both determined by plaque reduction assays), the compounds evaluated here fell into two groups. Group A had a cowpox EC_{50}/vaccinia EC_{50} ratio of approximately 0.4–0.8 and included compounds **1**, **3**, **4** and **5**. In other words, these compounds were almost twice as active against cowpox virus as against vaccinia virus. Group B consisted of agents that had a cowpox EC_{50}/vaccinia EC_{50} of 1 or greater. These included compound **2** and **cidofovir** with cowpox EC_{50}/vaccinia EC_{50} ratios of 1.2 and 1.7, respectively. These latter compounds were therefore less active against cowpox virus than against vaccinia virus. Whether these differences speak to some differences in mode of action remains to be established.

Compound **1** does not owe its activity to transformation to the parent 5-formyl-2′-deoxyuridine since the latter is without significant anti-orthopoxvirus activity (X. Fan, K.A. Keith, E.R. Kern and P.F. Torrence, unpublished observations). Moreover, the dimethylacetal **1** is quite stable in pH 7.5 buffer at 37 °C with a half-life approximating 2 weeks (X. Fan and P.F. Torrence, unpublished observations). The corresponding diethylacetal congener is nearly devoid of antiviral activity (P.F. Torrence, X. Fan, K.A. Keith and E.R. Kern, unpublished observations). When the dicyanovinyl congener **2** is modified to the corresponding monocyanovinyl analogue (mixture of *E* and *Z* isomers), it becomes devoid of anti-orthopoxvirus activity (P.F. Torrence, X. Fan, K.A. Keith and E.R. Kern, unpublished observations). As limited as these SAR results are at this time, they suggest, for compounds **1** and **2**, an intimate relationship between structure and anti-orthopoxvirus activity.

3. Conclusion

We have demonstrated herein and in studies yet to be published that much new chemistry and lead drug discovery lies in

the exploration of the chemical space presently vaguely defined as "the pyrimidine nucleoside 5-substituent". Their mechanisms of action, spectrum of antiviral activities and structure–activity relationships are under active investigations. We expect at minimum these novel nucleosides may provide additional insights into how viral diseases may be, if not conquered, at least controlled.

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