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Anti-coxsackievirus B3 activity of 2-amino-3-nitropyrazolo [1,5-*a*]pyrimidines and their analogs

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Abstract—A novel class of 2-amino-4-nitropyrazolo[1,5-*a*]pyrimidines has been identified as potent inhibitors of coxsackievirus B3 replication. The synthesis of these compounds is based on the regioselective reaction of 3,5-diamino-5-nitropyrazole with unsymmetrical β-diketones at catalysis by hydrochloric acid leading to 2-amino-4-nitropyrazolo[1,5-*a*]pyrimidines as key steps.

Picornaviruses, in particular enteroviruses and rhinoviruses, are responsible for the majority of human respiratory diseases.[1] Moreover, enteroviruses may cause aseptic meningitis,[2] encephalitis,[3] febrile illness, hand-foot-mouth disease,[4] and myocarditis.[5] The enterovirus, coxsackievirus B3 (CVB3) is considered to be the most important etiological agent of virus-induced acute and chronic myocarditis.[6,7] Because more than 65 enterovirus serotypes exist, it was not possible to generate a vaccine until now and considerable interest exists to develop antiviral compounds. So far, these virus infections are treated symptomatically because there are no virus-specific prophylactics and drugs available for clinical use.

In this paper, we discuss the synthesis of 2-amine-3-nitropyrazolo[1,5-*a*]pyrimidine derivatives and the results of investigations on their cytotoxic, antiproliferative, and antiviral activity. Based on these data the relationship between the chemical structure and cytotoxicity, cell growth (proliferation) inhibition, and anti-CVB3 activity was analyzed. A general synthetic route for the synthesis of the pyrazolopyrimidines is described in Scheme 1.

The 3,5-diamino-4-nitropyrazole was prepared via condensation of 1-dimethylamino-2-nitroenamine[8] **1** with hydrazine hydrate in ethanol under reflux conditions and recrystallization of pyrazole **2** from propanol-2. The condensations of pyrazole **2** with commercially available β-dicarbonyl compounds in methanol give rise to the 2-amino-3-nitropyrazolo[1,5-*a*]pyrimidines **3**,**4**. Generally, this reaction may result in two regioisomers (5-*R*-7-oxo and 7-oxo-5-*R*-pyrazolo[1,5-*a*]pyrimidine). However, the reactions selectively result in 2-amino-3-nitro-5-*R*-7-oxopyrazolo[1,5-*a*]pyrimidines under condition of catalysis by the methanol solution of hydrochloric acid or sodium hydroxide.[9] The subsequent reaction with exchange of chlorine atom in pyrazolopyrimidine **4** with different nucleophilic agents in presence of triethylamine under reflux condition results in a series of final compounds (**5a**–**m**).[10] The reaction was controlled by TLC (hexane/acetone: 3/1).

The compounds were examined in regard of cytotoxicity in human cervix carcinoma cells (HeLa), growth inhibition of mouse fibroblasts (L-929) as well as human chronic myeloid leukemic cells (K-562) and anti-CVB3 activity in HeLa cells. The results are presented in Table 1 and demonstrate a good compatibility of the test compounds for confluent as well as proliferating cells. None of the pyrazolo[1,5-*a*]pyrimidine derivatives inhibited the influenza virus A or herpes simplex virus type 1-induced cytopathic effect in Madin Darby canine kidney

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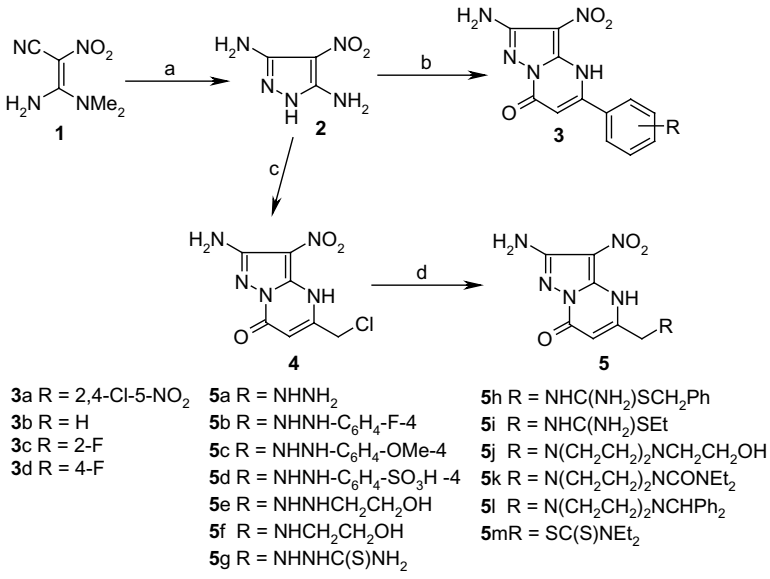

Scheme 1. Synthesis of 2-amino-3-nitropyrazolo[1,5-*a*]pyrimidine derivatives. Reagents and conditions: (a) NH_2NH_2 H_2O, EtOH, reflux, 86%; (b) ethyl *R*-benzoylacetic acid ester, MeOH, HCl/MeOH or NaOH, reflux, 90%; (c) ethyl 4-chloro-3-oxobutanoate, HCl/MeOH, MeOH, reflux, 83%; (d) nucleophylic agent, Et_3N, EtOH, reflux, 50–80%.

Table 1. 50% cytotoxic concentration (CC_{50}),[11] 50% growth inhibitory concentration (GI_{50}),[12] 50% inhibitory concentration determined with coxsackievirus B3 in HeLa cells (IC_{50}),[13] therapeutic index (TI),[13] and log *P*[15] of 2-amino-4-nitropyrazolo[1,5-*a*]pyrimidine

Compound	CC_{50} (μg/ml) in HeLa cells	GI_{50} (μg/ml) in		IC_{50} (μg/ml) CVB3	TI (CC_{50}:IC_{50})	Log *P*
		L-929 cells	K-562 cells			
3a	>100	>50	>50	3.94	>25.6	2.77 ± 0.27
3b	>100	>50	>50	29.60	>3.4	2.54 ± 0.09
3c	66.5	>50	>50	20.35	3.2	2.34 ± 0.16
3d	>100	>50	>50	50.29	>2.0	2.34 ± 0.16
5a	42.6	35.2	26.5	66.45	Not active	0.42 ± 0.09
5b	>100	41.4	19.7	5.81	>17.2	2.65 ± 0.22
5c	66.3	>50	5.1	17.53	3.8	2.46 ± 0.27
4, **5d–k**	>50	>50	>50	>50	Not active	
5l	14.8	19.9	9.0	>50	Not active	3.73 ± 0.16
5m	>50	>50	33.1	>50	Not active	3.06 ± 0.12

or green monkey kidney cells, respectively (results not shown). Six 2-amino-3-nitro-7-hydroxypyrazolo[1,5-*a*]-pyrimidine derivatives with a benzene moiety or hydrazinomethylene substitutes in position 5 of the pyrimidine ring (compounds **3a**–**d**, **5b**, **c**) exhibited a specific inhibitory effect against CVB3 (Fig. 1).

SARS studies revealed that only compounds containing a free 2-amino group and 3-nitro- and 7-oxo- substitutes showed antiviral activity. A complete loss of antiviral activity was observed if compounds with other than these substitutes were synthesized (structures not shown). Interestingly, the anti-CVB3 activity of another nitrocontaining compound, the 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile, was published during the preparation of this manuscript.[14] It exhibits broad-spectrum anti-picornavirus activity and inhibits CVB3 replication in primary human myocardial fibroblasts. Furthermore, the anti-CVB3 activity was completely lost after substitution of the phenyl group to piperasino, thiosemicarbasido, dithiocarbamato-groups or other moiety (compounds **4**, **5a**,**g**–**m**).

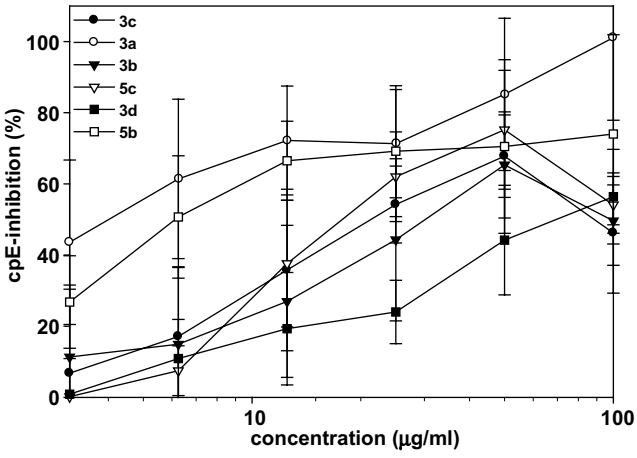

Figure 1. Mean anti-coxsackievirus B3 activity and standard deviation of pyrazolopyrimidines **3a**–**d**, **5b**, and **5c** determined in three independently performed CPE inhibitory assays.[13]

The comparison of SAR of our 2-amino-3-nitropyrazolo[1,5-*a*]pyrimidine derivatives with that of polysubstituted pyrazolo[3,4-*d*]pyrimidines described by J. H. Chern et al. in 2004[16] indicates that the introduction of phenyl substitutes led to an increased antiviral activity of both classes of compounds. But, whereas the most active polysubstituted pyrazolo[3,4-*d*]pyrimidines contained a hydrophobic diarylmethyl group at the piperasine, the same substitute caused a complete loss of antiviral activity when introduced into pyrazolo[1,5-*a*]-pyrimidine (compound **5l**).

Interestingly, a strong correlation between antiviral activity and the index of lipophilicity[15] of active compounds was found. It was established, that compounds with log *P* 2.2–2.8 range were active, whereas more lipophilic or hydrophilic compounds were inactive.

In summary, a novel class of potent coxsackievirus B3 inhibitors was synthesized and evaluated. A method of regioselective condensation of 3,5-diamino-4-nitropyrazole **2** with β-diketones was elaborated resulting selectivity in the synthesis of 2-amino-3-nitro-5-*R*-7-oxo-pyrazolo[1,5-*a*]pyrimidines. The synthetic routes are brief and scalable, allowing of rapid analogs preparation.

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11. To determine the 50% cytotoxic concentration (CC_{50}), confluent HeLa cell monolayer grown in 96-well plates were incubated with serial dilutions (factor 2, each concentration in duplicate) of the respective compounds for 72 h (37 °C, 5% CO_2). Then, the cells were fixed and stained with a crystal violet formalin solution. Cytotoxicity was quantified spectrophotometrically with a plate reader as described previously Schmidtke, M.; Schnittler, U.; Jahn, B.; Dahse, H.-M.; Stelzner, A. *J. Virol. Methods* **2001**, *95*, 133.
12. 1×10^4 K-562 or L-929 cells were added to 10 dilutions (dilution factor 2) of test compounds. After an incubation time of 72 h (37 °C, 5% CO_2) the proliferation of suspension cultures of K-562 cells were analyzed by an electronic cell analyzer system (CASY) whereas the growth of the adherent L-929 cells was studied by determination of optical density (OD) staining with methylene blue.
13. The cytopathic effect (CPE) inhibitory assay was performed as described previously.[11] Briefly, 50 μl of drug solution and 50 μl of a constant amount of virus (1 MOI for CVB3, 0.1 MOI for influenzavirus A, and 0.1 MOI for HSV-1 K1) were added to confluent HeLa, MDCK, or GMK cell monolayers, respectively, grown in 96-well plates. The inhibition of the virus-induced CPE was scored spectrophotometrically 24 h (CVB3 and influenza virus A) or 48 h (HSV-1 K1) post infection when untreated infected control cells showed maximum cytopathic effect. The therapeutic index (TI) was calculated by dividing the mean CC_{50} by the mean 50% inhibitory concentration (IC_{50}) of a test compound.
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