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Activity of 6-aryl-pyrrolo[2,3-*d*]pyrimidine-4-amines to *Tetrahymena*

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ABSTRACT

A series 6-aryl-pyrrolo[2,3-*d*]pyrimidine-4-amines (43 compounds), some of which are epidermal growth factor tyrosine kinase inhibitors, were tested for their protozoal toxicity using an environmental *Tetrahymena* strain as model organism. The protozoacidal activity of the analogues was found to be highly dependent on a 4-hydroxyl group at the 6-aryl ring, and a chiral 1-phenylethanamine substituent in position 4. Further, the potency was affected by the aromatic substitution pattern of the phenylethanamine: the unsubstituted, the *meta*-fluoro and the *para*-bromo substituted derivatives had the lowest minimum protozoacidal concentrations (8–16 μg/mL). Surprisingly, both enantiomers were found to have high potency suggesting that this compound class could have several modes of action. No correlation was found between the compounds protozoacidal activity and the *in vitro* epidermal growth factor receptor tyrosine kinase inhibitory potency. This suggests that the observed antimicrobial effects are related to other targets. Testing towards a panel of kinases indicated several alternative modes of action.

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1. Introduction

Diseases caused by parasitic protozoa, as for instance malaria, dysentery, leishmaniasis, and human African trypanosomiasis are major causes of mortality throughout the world, thus, the study of effects of organic compounds on protozoa is important. Therapeutic agents are available [1,2] however, many of the drugs have critical side effects [3,4] and also resistance is emerging [5,6]. Therefore, identification of new lead compounds is required, and inhibition of cellular kinase activity has been recognised as a useful strategy [7–11]. Among others, tyrosine kinase inhibitors such as Erlotinib, Canertinib and Sunitinib designed for cancer chemotherapy have been identified as efficient antiprotozoal agents [11].

Tetrahymena is a genus of ciliated protozoa. Its members are easily grown and relatively safe to handle making them useful model systems for biochemical mechanistic studies in eukaryotes [12]. The motility behaviour of *Tetrahymena* is conveniently used to monitor bioactivity and cell toxicity of chemicals [13–15]. Compounds such as diphenols, aminophenols, diaminoaromatics, halogenated aromatic nitro compounds, aromatic aldehydes and α-haloketones are generally toxic to *Tetrahymena*. This is due to their ability to undergo various reactions with biomacromolecules [16–19]. *Tetrahymena* do not pose a serious threat to human health. However, *Legionella* in symbiosis with *Tetrahymena tropicalis* appears more resistant and aggressive [20,21]. In addition, infections attributed to members of this genus are a problem in closed fish farming. Low molecular weight compounds such as Menadione (**I**) [22], and anti-infective agents such as Niclosamide (**II**) have been proposed as treatment alternatives [23]. Other compounds with *in vitro* activity towards *Tetrahymena* include among others Climacostol (**III**) [24], known antimicrobial agents as Chloroquine [25] and Chloroamphenicol [26,27] and antineoplastics such as Necodazole [28], Fig. 1.

Tetrahymena are known to have epidermal growth factor (EGF)-like receptors which are involved in cell division [29], and cyst formation [30]. Also other processes such as chemotaxis [31], hormonal imprinting [32], cell division [33,34], stress response [34,35], and GTP signalling [36], are triggered and controlled by kinase activity. Using an environmental *Tetrahymena* isolate as model, we have evaluated the potency of a series of 6-aryl-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-amines as antiprotozoal agents. One goal has been to identify new lead compounds for combating protozoa. Secondly, the study could shed light on the toxicity profile of this compound class since some of the derivatives are efficient inhibitors of the epidermal growth factor receptor tyrosine kinase (EGFR-TK) *in vitro* [37].

2. Materials and methods

2.1. General

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 400 spectrometer operating at 400 MHz and 100 MHz,

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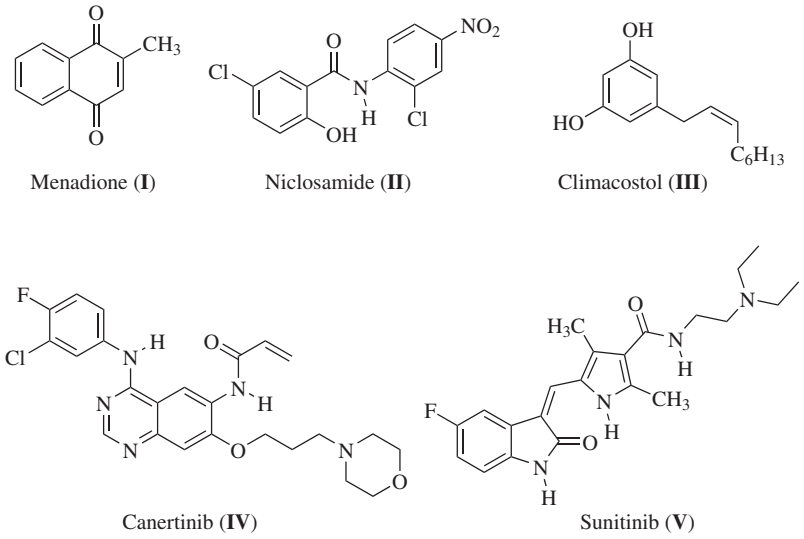

Fig. 1. Structure of compounds **I–III** with potency towards *Tetrahymena* and the kinase inhibitors Canertinib (**IV**) and Sunitinib (**V**).

respectively. ^{19}F NMR was performed on a Bruker Avance 600 operating at 564 MHz. The ^{19}F NMR shift values are relative to hexafluorobenzene. Coupling constants are in Hertz. HPLC (Agilent 110-Series) with a G1379A degasser, G1311A Quatpump, G1313A ALS autosampler and a G1315D Agilent detector (230 nm) was used to determine the purity of the synthesised compounds. Conditions: a Omrisphere 5 C18 (100 × 3.0 mm) column, flow rate 1.0 mL/min, elution starting with H_2O + 1% TFA/acetonitrile (98/2), linear gradient elution for 15 min. ending at acetonitrile/water+1% TFA (90/10), then 15 min isocratic elution. The software used with the HPLC was Agilent ChemStation. Accurate mass determination was performed with EI (70 eV) using a Finnigan MAT 95 XL. FTIR spectra were recorded on a Thermo Nicolet Avatar 330 infrared spectrophotometer. All melting points are uncorrected and measured by a Büchi melting point instrument. Optical rotation was measured with a PerkinElmer Instruments Model 341 Polarimeter.

2.2. Isolation and characterisation of Tetrahymena

The *Tetrahymena* strain used was originally isolated from pond water in Norway and was identified to the genus level based on its phenotype and on partial sequencing of the 18S rDNA-gene. The sequence had 100% identity with reported sequences for *Tetrahymena iwoffi*, *Tetrahymena tropicalis* and *Tetrahymena furgosoni*. The strain was maintained on non-nutrient agar (CCAP, Scotland) seeded with a thick suspension of pasteurised *Escherichia coli* prior to testing. The strain and further information on the sequencing studies can be made available on request.

2.3. Determination of minimum protozoacidal (MPC) concentrations

Stock solutions of the agents were made in DMSO at a concentration of 5120 μg/mL. Benzalkonium chloride (stock in water) was included as control. Water was used as dilutant producing doubling concentrations of the agents at 128–4 μg/mL. These intermediate dilutions (50 μL) were pipetted in triplicate into a 96-well, Nunc® round-bottomed microtiter plate system (Thermo Fischer Scientific, USA). Addition of 50 μL of the inoculum gave the final tested concentration range (2–64 μg/mL) and maximally 1.25% DMSO. A positive control (no agent), and a negative control (without Tetrahymena) tests were also included. *Tetrahymena* was grown on NNA seeded with a thick pasteurised suspension of *E. coli* for 48 h under a humidified atmosphere in the dark at 22 ± 2 °C. After incubation, protozoa were harvested and washed as previously described [38], and resuspended in pasteurised *E. coli* (corresponding to a MacFarland 0.5 standard) at 1×10^4 cells/mL. After incubation for 48 h at 22 ± 2 °C, wells were examined for motile cells using an inverted microscope. This approach enabled the whole content of the well to be visualised. The estimated minimum protozoacidal concentration (MPC; 48 h) was the lowest concentration at which no motile cells were seen. After examination in the microscope, the whole content of wells was transferred to culture dishes containing NNA/pasteurised *E. coli*. Cultures were examined over a 7-day period with an inverted microscope to see if a cell population developed. The MPC value measured (MPC; 7 days) was the lowest concentration that prevented the development of even a single viable cell in the 7-day period. Each test was performed in triplicate and the results were averaged to give the MPC value.

2.4. Kinase profiling

Compound (*R*)-**25e** was profiled utilising a panel of 124 protein kinases in the MRC National Centre for Protein Kinase Profiling Service at the University of Dundee (http://www.kinase-screen.mrc.ac.uk). The compound was tested *in vitro,* in duplicate, at a final concentration of 50 nM. For further details of the methodology see Bain et al. [39].

2.5. Synthesis

Detailed description of the synthesis and characterisation of most of the intermediates and tested compounds can be found elsewhere [37,40]. The synthesis and characterisation of the new chemical entities are given below.

2.5.1. General procedure thermal amination to ***20–24***

The following is representative: 4-chloro-6-(4-methoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (**14**) (275 mg, 1.06 mmol) and (*S*)-1-phenylethanamine ((*S*)-**19i**) (0.44 mL, ~3.5 mmol) were added to a dry round bottle flask containing 1-butanol (3.5 mL) under argon atmosphere. The mixture was heated at 145 °C for 24 h. The precipitate formed upon cooling to rt. was isolated by filtration, washed with diethyl ether (25 mL) and dried resulting in a solid.

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