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

Svein Jacob Kaspersen[a)], Eirik Sundby[b)], Colin Charnock[c)] Bård Helge Hoff [a)*].

[a)]Norwegian University of Science and Technology, Høgskoleringen 5, NO-7491 Trondheim, Norway.
[b)]Sør-Trøndelag University College, E. C. Dahls gate 2, 7004 Trondheim, Norway.
[c)]Oslo and Akershus University College of Applied Sciences, Postbox 4, St. Olavs plass, 0130 Oslo, Norway.

*Corresponding author: Bård Helge Hoff, fax: +0047 73544256, phone: +0047 73593973, E-mail: bard.helge.hoff@chem.ntnu.no.

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.

Keywords: Pyrrolopyrimidine; antiprotozoal agent; *Tetrahymena*; kinase; benzylamine,

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] Figure 1.

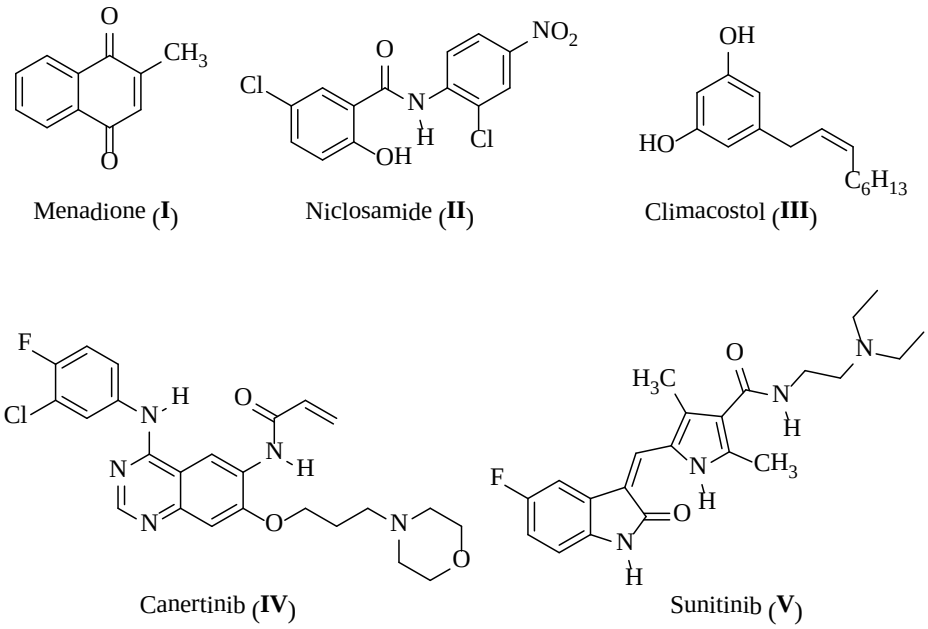


Figure 1. Structure of compounds **I-III** with potency towards *Tetrahymena* and the kinase inhibitors Canertinib (**IV**) and Sunitinib (**V**).

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, 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 (70eV) 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 *T. iwoffi*, *T. tropicalis* and *T. furgosoni*. The strain was maintained on non-nutrient agar (CCAP, Scotland) seeded with a thick suspension of pasteurised *E. 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 to 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 *n*M. 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 ^{o}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.

2.5.1.1 (*S*)-6-(4-Methoxyphenyl)-*N*-(1-phenylethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*S*)-20e)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**14**) (159 mg, 0.61 mmol) and (*S*)-1-phenylethanamine (**19e**) (222 mg, 1.84 mmol). This gave 179 mg (0.52 mmol, 85 %) of a white solid, mp. 226-228 °C, $[a]_D^{20}$ = +289 (*c* 0.17, DMSO). Spectroscopic properties were in correspondence with that reported previously for the (*R*)-enantiomer.[40] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.92 (s, 1H, NH, H-7), 8.04 (s, 1H, H-2), 7.73 (m, 3H), 7.43 (m, 2H), 7.30 (m, 2), 7.19 (m, 1H), 7.02 (d, J=8.8, 2H), 6.96 (bs, 1H, H-5), 5.50 (m, 1H), 3.80 (s, 3H), 1.53 (d, J= 7.0, 3H). HRMS (EI): 344.1634 (calcd $C_{21}H_{20}N_4O$, 344.1632, M^+).

2.5.1.2 (*R*)-6-(4-Methoxyphenyl)-*N*-(1-(4-methoxyphenyl)ethyl)-7H-pyrrolo[2,3-*d*]pyrimidin-4-amine ((*R*)-20h)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-metoxyphenyl)-7*H*-pyrrolo-[2,3-*d*]-pyrimidine (**14**) (238 mg, 0.92 mmol) and (*R*)-(4-methoxyphenyl)ethanamine (**19h**) (288 mg, 1.90 mmol). This gave 220 mg (0.59 mmol, 64%) of an off-white solid, mp. 249-251°C, $[a]_D^{20}$ = -330.1 (c 0.14, DMSO), purity > 99% (by HPLC). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.89 (s, 1H, NH, H-7), 8.04 (s, 1H), 7.71 (d, J=8.9, 2H), 7.64 (d, J=8.4, 1H, NH), 7.34 (d, J=8.7, 2H), 7.02 (d, J=8.9, 2H), 6.94 (s, 1H, H-5), 6.86 (d, J=8.7, 2H), 5.45 (m, 1H), 3.80 (s, 3H), 3.71 (s, 3H), 1.50 (d, J=7.0, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 158.6, 157.9, 154.8, 151.3 (2C), 137.5, 133.4, 127.2 (2C), 125.9 (2C), 124.5, 114.4 (2C), 113.5 (2C), 103.9, 94.6, 55.2, 55.0, 48.0, 22.9. HRMS (ESI): 375.1814 (calcd $C_{22}H_{22}N_4O_2$, 375.1816, $M+H^+$). IR (neat, cm^{-1}): 3099, 2973, 1588, 1244, 830.

2.5.1.3 (*S*)-N-(1-(4-Bromophenyl)ethyl)-6-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*S*)-20i)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-methoxyphenyl)-7*H*-pyrrolo-[2,3-*d*]-pyrimidine (**14**) (190 mg, 0.73 mmol) and (S)-1-(4-bromophenyl)ethanamine (**19i**) (439 mg, 2.19 mmol). This gave 245 mg (0.58 mmol, 79%) of a white solid, mp. 274-275 °C, $[a]_D^{20}$ = +309 (*c* 0.21, DMSO). Spectroscopic properties were in correspondence with that reported previously for the (*R*)-enantiomer.[37] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.93 (s, 1H, NH, H-7), 8.03 (s, 1H, H-2), 7.76 (s, 1H, NH), 7.73 (m, 2H), 7.49 (m, 2H), 7.38 (m, 2H), 7.02 (m, 2H), 6.94 (d, J=1.8, 1H, H-5), 5.44 (m, 1H), 3.80 (s, 3H), 1.51 (d, J=7.0, 3H). HRMS (EI): 422.0739 (calcd $C_{21}H_{19}BrN_4O$, 422.0737, M^+)

2.5.1.4 6-(4-Methoxyphenyl)-N-(1-naphthalen-1-ylmethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (20o)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**14**) (154 mg, 0.59 mmol) and naphthalen-1-ylmethanamine (**19o**) (280 mg, 1.78 mmol). This gave 173 mg (0.45 mmol, 77%) of a white solid, mp 278-281°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H, NH, H-7), 8.21-8.19 (m, 1H), 8.14 (s, 1H, H-2), 7.97-7.94 (m, 2H), 7.86-7.84 (m, 1H), 7.71-7.69 (m, 2H), 7.57-7.52 (m, 3 H), 7.49-7.46 (m, 1H), 7.01-7.00 (m, 2H), 6.89 (s, 1H, H-5), 5.20 (d, J=5.6, 2H), 3.79 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6), δ: 159.1, 155.8, 151.8 (2C, overlap), 135.7,

134.2, 133.8, 131.5, 128.9, 127.8, 126.6, 126.4 (2C), 126.2, 125.9, 125.7, 124.9, 124.0, 114.9 (2C), 104.4, 95.0, 55.6, 41.9. IR (neat, cm^{-1}): 3152, 1597,1254,769. HRMS (EI): 380.1632 (calcd $C_{24}H_{20}N_4O$, 380.1632, M^+)

2.5.1.5 (*R*)-6-(4-Methoxyphenyl)-*N*-(1-(naphthalen-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*R*)-20p)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidine (**14**) (144 mg, 0.55 mmol) and (*R*)-1-(naphthalen-1-yl)ethanamine (**19p**) (285 mg, 1.66 mmol). This gave 162 mg (0.41 mmol, 74%) of a white solid, mp. 274-276 °C, $[a]_D^{20}$ = -432 (*c* 0.37, DMSO). Spectroscopic properties were in correspondence with that reported previously for the racemate.[37] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.93 (s, 1H, NH, H-7), 8.24 (d, J=8.2, 1H), 8.04 (s, 1H, H-2), 7.94 (m, 1H), 7.87 (m, 1H), 7.81 (m, 1H), 7.71-7.64 (m, 3H), 7.59-7.45 (m, 3H), 7.01 (m, 2H), 6.97 (s, 1H, H-5), 6.27 (m, 1H), 3.79 (s, 3H), 1.67 (d, J=6.8, 3H). IR (neat, cm^{-1}): 3131, 2962 1624, 1251, 828, 775. HRMS (EI): 394.1783 (calcd $C_{25}H_{22}N_4O$, 394.1794, M^+).

2.5.1.6 (*R*)-N-(1-(Naphthalen-1-yl)ethyl)-6-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*R*)-21p)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-phenyl)-7H-pyrrolo[2,3-d]pyrimidine (**15**) (87 mg, 0.38 mmol) and (R)-1-(naphthalen-1-yl)ethanamine (**19p**) (195 mg, 1.14 mmol). This gave 107 mg (0.29 mmol, 76%) of a white solid. The solid melted at 155-157 °C, but partly re-solidified to a solid melting at 200°C, $[a]_D^{20}$ = -481 (*c* 1.00, DMSO), purity: 98% (by HPLC). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.07 (s, 1H, NH, H-7), 8.26-8.24 (m, 1H), 8.08 (s, 1H, H-2), 8.01-7.93 (m, 2H), 7.82-7.77 (m, 3H), 7.67-7.66 (m, 1H), 7.59-7.41 (m, 5H), 7.30-7.27 (m, 1H), 7.14 (s, 1H, H-5), 6.32-6.25 (m, 1H), 1.68 (d, J=6.8, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 155.3, 152.3, 152.0 141.3, 133.92, 133.87, 132.3, 131.1, 129.42 (2C), 129.1, 127.7, 127.6, 126.6, 126.0, 125.9, 124.9 (2C), 123.8, 122.7, 104.4, 60.2, 96.7, 22.3. IR (neat, cm^{-1}): 2976, 1586, 1471, 1311, 774, 749. HRMS (EI): 364.1683 (calcd $C_{24}H_{20}N_4$, 364.1682, M^+).

2.5.1.7 (*S*)-N-(1-(Naphthalen-1-yl)ethyl)-6-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*S*)-21p)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-phenyl)-7H-pyrrolo[2,3-d]pyrimidine (**15**) (70 mg, 0.30 mmol) and (*S*)-1-phenylethanamine (**19e**) (156 mg, 0.91 mmol). This gave 84 mg (23 mmol, 77%) of a white solid, mp. 162-166 °C, $[a]_D^{20}$ = +422 (*c* 1.00, DMSO), purity: 98% (by HPLC). The spectroscopic properties corresponded with that reported for (*S*)-**21p** in section 2.5.1.6. HRMS (EI): 364.1682 (calcd $C_{24}H_{20}N_4$, 364.1682, M^+).

2.5.1.8 (*R*)-6-(4-Fluorophenyl)-N-(1-(naphthalen-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*R*)-22p)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-fluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**16**) (64 mg, 0.26 mmol) and (*R*)-1-(naphthalen-1-yl)ethanamine (**19p**) (133 mg, 0.78 mmol). This gave 70 mg (0.18 mmol, 70%) of a white solid, mp. 147-150 °C, $[a]_D^{20}$ = -444 (*c* 0.40, DMSO), purity: 99% (by HPLC). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.07 (s, 1H, NH, H-7), 8.26-8.24 (m, 1H), 8.08 (s, 1H, H-2), 7.99-7.93 (m, 2H), 7.82-7.80 (m, 3H), 7.67-7.65 (m, 1H), 7.58-7.45 (m, 3H), 7.31-7.27 (m, 2H), 7.09 (s, 1H, H-5), 6.31-6.25 (m, 1H), 1.68 (d, J=6.8, 3H). ^{13}C NMR (100 MHz, DMSO-d_6), δ: 161.9 (d, J=244.5), 155.3, 152.3 (2C), 152.0, 141.3, 139.9, 133.0, 131.1, 129.1, 128.9 (d, J=2.9),

127.6, 126.9 (d, J=7.9), 126.6, 126.0 (d, J=3.5, 2C), 123.8, 122.7, 116.4 (d, J=21.7, 2C), 104.4, 96.6, 60.2, 22.2. ^{19}F NMR (564 MHz, DMSO-d_6, C_6F_6) δ: -117.1 (m). IR (neat, cm^{-1}): 2985, 1585, 1496, 1312, 1233, 834, 774. HRMS (EI): 382.1585 (calcd $C_{24}H_{19}FN_4$, 382.1586, M^+).

2.5.1.9 (*S*)-6-(4-Fluorophenyl)-N-(1-(naphthalen-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*S*)-22p)

The compound was prepared as described in Section 2.5.1 starting with 4-chloro-6-(4-fluorophenyl)-7H-pyrrolo[2,3-d]pyrimidine (**16**) (66 mg, 0.27 mmol) and (*S*)-1-phenylethanamine (**19p**) (137 mg, 0.80 mmol). This gave 76 mg (0.20 mmol, 74%) of a white solid, mp. 149-152 °C, $[a]_D^{20}$ = +380 (*c* 0.13, DMSO), purity: 98% (by HPLC). The spectroscopic properties corresponded with that reported for (*R*)-**22p** in section 2.5.1.8.

2.5.2 General procedure demethylation of 20 to 25

The following is representative: (*S*)-6-(4-methoxyphenyl)-*N*-(1-phenylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**20i**) was dissolved in dry CH_2Cl_2 (2 mL) under argon atmosphere. BBr_3 (0.17 ml, ~1.8 mmol) in dry CH_2Cl_2 (1.5 mL) was added drop wise over 1 h. at 0 °C using a syringe pump. Then the mixture was allowed to react at 20 °C for 24 h. The reaction was quenched by addition of water (10 mL), and the mixture was extracted with EtOAc (3×25 mL). The combined organic phase was washed with brine (15 mL), dried over $MgSO_4$ and concentrated. The resulting residue was purified by precipitation from acetone (0.5 mL). The solid formed was isolated by filtration, washed with diethyl ether (10 mL) and dried.

2.5.2.1 (*S*)-4-(4-(1-Phenylethylamino)-7H-pyrrolo[2,3-d]pyrimidin-6-yl)phenol hydrobromide ((*S*)-25e)

The compound was synthesised as described in Section 2.5.2 starting from (*S*)-6-(4-methoxyphenyl)-*N*-(1-phenylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**20e**) (99 mg, 0.29 mmol). This gave 56 mg, (0.14 mmol, 47%) of a white solid, mp > 300 °C, $[a]_D^{20}$ = +289 (*c* 0.17, DMSO). purity > 98% (by HPLC) Spectroscopic properties were in correspondence with that reported previously for the (*R*)-enantiomer.[37] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.98 (s, 1H, NH, H-7), 9.83 (br s, 1H, OH), 9.55 (br s, 1H, NH), 8.30 (s, 1H, H-2), 7.66 (m, 2H), 7.50-7.48 (m, 2H), 7.39 (m, 2H), 7.32-7.29 (m, 1H), 7.24 (s, 1H, H-5), 6.89 (m, 2H), 5.37 (m, 1H), 1.66 (d, J=6.5, 3H). HRMS (EI): 330.1475 (calcd $C_{20}H_{18}N_4O$, 330.1475, M^+).

2.5.2.2 (*S*)-4-(4-(1-(4-bromophenyl)ethylamino)-7H-pyrrolo[2,3-d]pyrimidin-6-yl)phenol hydrobromide ((*S*)-25i)

The compound was synthesised as described in Section 2.5.2 starting from (*S*)-*N*-(1-(4-bromophenyl)ethyl)-6-(4-methoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**20i**) (134 mg, 0.32 mol). This gave 83 mg (0.17 mmol, 52%) of a white solid, mp. 274-276 °C, $[a]_D^{20}$ = +272 (*c* 0.28, DMSO), purity > 97% (by HPLC). Spectroscopic properties were in correspondence with that reported previously for the (*R*)-enantiomer.[37] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.94 (s, 1H, NH, H-7), 9.82 (br s, 1H, OH), 9.47 (br s, 1H, NH), 8.29 (s, 1H, H-2), 7.65 (m, 2H), 7.58 (m, 2H), 7.44 (m, 2H), 7.16 (s, 1H, H-5), 6.88 (m, 2H), 5.37 (m, 1H), 1.63 (d, J=6.6, 3H. HRMS (EI): 408.0581 (calcd $C_{20}H_{17}Br^{79}N_4O$, 408.0580, M^+).

2.5.2.3 4-(4-(Naphthalen-1-ylmethylamino)-7H-pyrrolo[2,3-d]pyrimidin-6-yl)phenol hydrobromide (25o)

The compound was synthesised as described in Section 2.5.2 starting from 6-(4-methoxyphenyl)-*N*-(naphthalen-1-ylmethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**20o**) (117 mg, 0.31 mmol) and BBr_3 (0.29 mL, 3.1 mmol). This gave 74 mg (0.20 mmol, 66%) of a white solid, mp > 300 °C. purity > 98% (by HPLC). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.01 (s, 1H, H-7), 9.82 (s, 1H, OH), 8.36 (s, 1H, H-2), 8.15-8.13 (m, 1H), 8.04-8.02 (m, 1H), 7.97-7.95 (m, 1H), 7.66-7.60 (m, 4H), 7.54-7.7.50 (m, 2H), 7.17 (s, 1H, H-5), 6.87-6.89 (m, 2H), 5.26 (bs, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 158.5, 149.9, 148.6, 142.8, 138.1, 133.9, 132.1, 131.3, 129.1, 128.9, 127.2 (2C), 127.1, 126.7, 126.1, 125.4, 124.1, 121.7, 116.4 (2C), 103.5, 96.8, 43.9. IR (neat, cm^{-1}): 3123, 1643, 1612, 1493, 1178, 757. HRMS (EI): 366.1471 (calcd $C_{23}H_{18}N_4O$, 366.1475, M^+).

2.5.2.4 (*R*)-4-(4-(1-(naphthalen-1-yl)ethylamino)-7H-pyrrolo[2,3-d]pyrimidin-6-yl)phenol ((*R*)-25p)

The compound was synthesised as described in Section 2.5.2 starting (R)-6-(4-methoxyphenyl)-*N*-(1-(naphthalen-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*R*)-**20p**) (113 mg, 0.29 mmol). This gave 58 mg (0.15 mmol, 53%) of a white solid, mp > 300 °C. $[a]_D^{20}$ = -379 (*c* 0.26, DMSO) purity > 98% (by HPLC). Spectroscopic properties were in correspondence with that reported previously for the racemate.[37] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.98 (s, 1H, NH, H-7), 9.82 (br s, 1H, OH), 9.55 (br s, 1H, NH), 8.30 (s, 1H), 8.14 (s, 1H, H-2), 8.00 (m, 1H), 7.91 (m, 1H), 7.64-7.57 (m, 5H), 7.54-7.50 (m, 1H), 7.26 (s, 1H, H-5), 6.89-6.87 (m, 2H), 6.09 (m, 1H), 1.78 (d, J=6.5, 3H). HRMS (EI): 380.1635 (calcd $C_{24}H_{20}N_4O$, 380,1637, M^+).

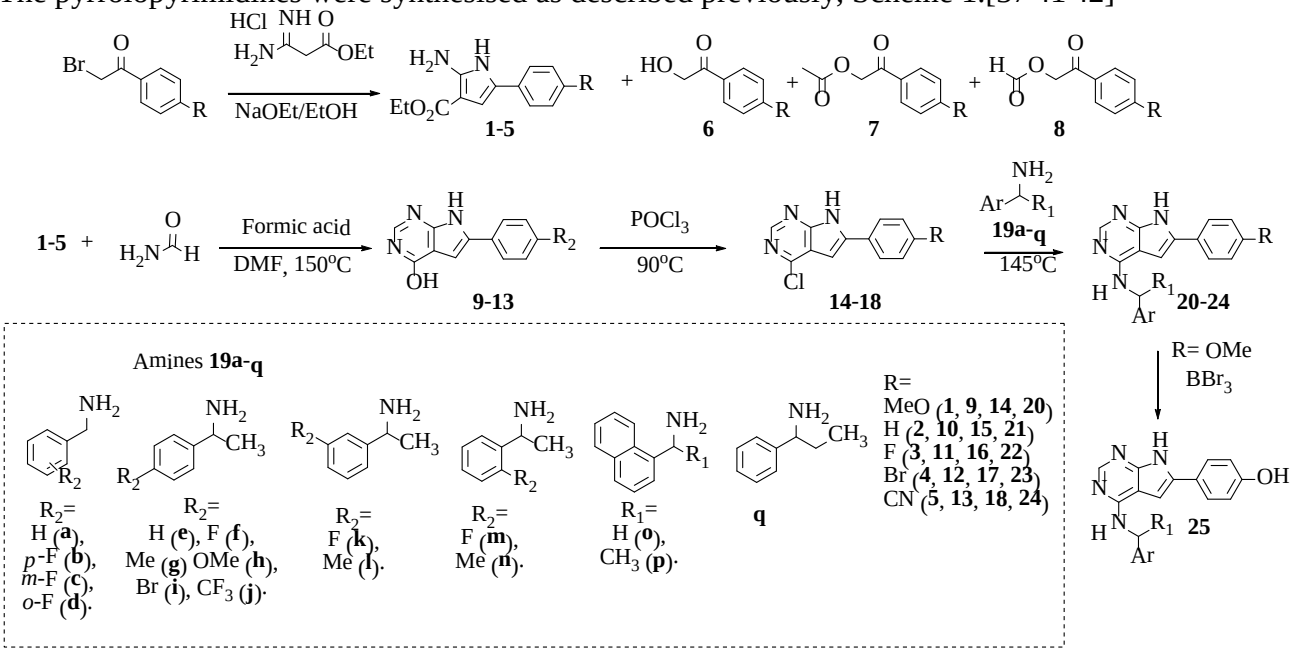
2.5.2.5 (*S*)-4-(4-(1-(Naphthalen-1-yl)ethylamino)-7H-pyrrolo[2,3-d]pyrimidin-6-yl)phenol hydrobromide ((*S*)-**25p**)

The compound was synthesised as described in Section 2.5.2 starting (S)-6-(4-methoxyphenyl)-N-(1-(naphthalen-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine ((*S*)-**20p**) (73 mg, 0.19 mmol). This gave 48 mg (0.13 mmol, 68%) of a white solid, mp > 300 °C. $[a]_D^{20}$ = +389 (*c* 0.16, DMSO) purity > 99% (by HPLC). Spectroscopic properties were in correspondence with that reported previously for the racemate.[37] and that described for (*R*)-**25p** in section 2.5.2.4.

3. Result and Discussion

3.1 Synthesis

The pyrrolopyrimidines were synthesised as described previously, Scheme 1.[37,41,42]



Scheme 1. Synthesis of **20-25** using the amines **19a-q.**

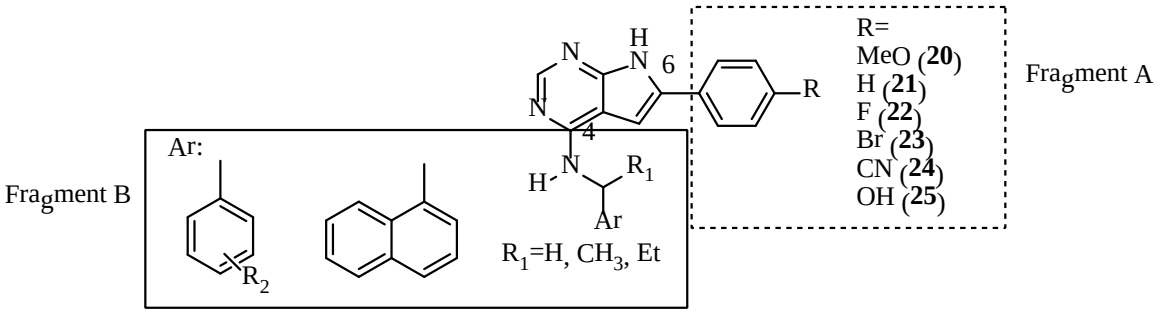
The first step forming the pyrroles **1-5** gave mediocre yields. We found that formation of **6-8** was the main reason for the loss in yield. The alcohol **6** may be caused by water generated in the pyrrole cyclisation, whereas the esters **7** and **8** most likely originate from fragmentation of the unstable aminoimidate. An increase in yield of **1-5** was seen when using >2 equivalents of the aminoimidate and 3 equivalents of sodium ethoxide. It has previously been suggested that such pyrroles are UV labile.[42] Discolouration of the products was seen on storage in DMSO for one day at room temperature. Cyclisation of **1-5** using formamide gave the 4-hydroxypyrrolopyrimidines **9-13** which all were crystalline and easily isolated. Standard chlorination gave **14-18**, which also were conveniently isolated and purified if full conversion was obtained in the reaction. In the next step, thermal nucleophilic aromatic substitution on **14-18** was performed using various amines. Compound **14** was reacted with **19a-q** giving **20a-q**, while the 4-chloropyrrolopyrimidines **15-18** were mainly substituted with **19e-f** and **19p**, giving the corresponding 4-amino derivatives **21-24**. Deprotection of the methoxy derivatives **20** with boron tribromide gave the phenolic compounds **25**. Twelve of the compounds reported in this study are new chemical entities.

3.2 Toxicity towards *Tetrahymena*

The *Tetrahymena* strain used in this study was originally isolated from pond water in Norway. It was observed that this isolate grew faster and was more vigorous than several of our culture collection *Tetrahymena* strains. These observations were considered important when choosing it as a test strain for the present study. Benzylalkonium chloride was used as a control in the testing showing a MPC value of 8 µg/mL. We first investigated the effect of compounds having benzylamines, chiral 1-phenylethanamines and 1-naphthylethanamines as substituents

in Fragment B (see Table 1), and methoxy, hydrogen, fluoro, bromo and cyano as R in Fragment A.

Table 1. Activity of the pyrrolopyrimidines **20-24** and **25o-p** towards *Tetrahymena*.



Entry	Substance	R	R_1	Ar/R_2	MPC µg/mL (48 h)[a)]
1	**20a**	OMe	H	H	>64
2	**20b**	OMe	H	*p*-F	>64
3	*(R)*-**20e**	OMe	CH_3	H	>64
4	(*S*)-**20e**	OMe	CH_3	H	>64
5	*(R)*-**20h**	OMe	CH_3	*p*-MeO	>64
6	**20o**	OMe	H	$C_{10}H_7$	>64
7	*(R)*-**20p**	OMe	CH_3	$C_{10}H_7$	32[b)]
8	*(S)*-**20p**	OMe	CH_3	$C_{10}H_7$	>64
9	*(R)*-**20r**	OMe	Et	H	>64
10	**21c**	H	H	*m*-F	>64
11	**21e**	H	CH_3	H	>64
12	*(R)*-**21f**	H	CH_3	*p*-F	>64
13	*(R)*-**21p**	H	CH_3	$C_{10}H_7$	>64
14	(*S*)-**21p**	H	CH_3	$C_{10}H_7$	>64
15	**22c**	F	H	*m*-F	>64
16	*(R)*-**22e**	F	CH_3	H	>64
17	*(R)*-**22f**	F	CH_3	*p*-F	>64
18	(*R*)-**22p**	F	CH_3	$C_{10}H_7$	>64
19	(*S*)-**22p**	F	CH_3	$C_{10}H_7$	>64
20	*(R)*-**23e**	Br	CH_3	H	>64
21	*(R)*-**23f**	Br	CH_3	p-F	>64
22	*(R)*-**24e**	CN	CH_3	H	>64
23	*(R)*-**24f**	CN	CH_3	*p*-F	>64
24	**25o**	OH	H	$C_{10}H_7$	64
25	(*rac*)-**25p**	OH	CH_3	$C_{10}H_7$	64
26	(*R*)-**25p**	OH	CH_3	$C_{10}H_7$	32[a)]
27	(*S*)-**25p**	OH	CH_3	$C_{10}H_7$	64

[a)] The MPC values were determined by averaging three parallel measurements. [b)] MPC =32 also after 7 days.

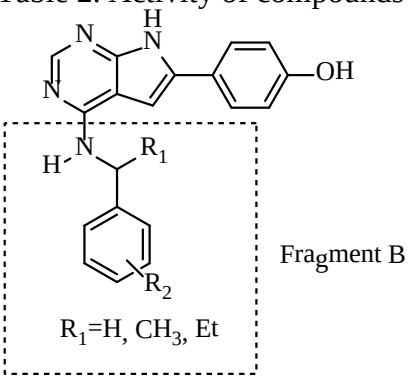
Most of the compounds were synthesised as their (*R*)-enantiomers, but the (*S*)-enantiomers of the methoxy substituted compound **20e**, and **20p**, **21p** and **22p** having a 1-naphthylethanamine substituent at C-4 were included to investigate the importance of stereochemistry. Testing revealed that the (*R*)-naphthyl derivative **20p** had an MPC-value of

32 (Table 1, entry 7), while other derivatives with R=OMe, H, F, Br and CN did not possess protozoacidal activity with respect to *Tetrahymena* in the lower µg/mL concentration range.

Solubility challenges were encountered when some of these compounds were tested, the investigation was continued with the naphthylic compounds **25o-p** which had a hydrophilic phenolic group in fragment A, (Table 1, entries 24-27). However, no drastic improvement in potency was observed. The (*R*)-enantiomer of **25p** (Table 1, entry 26) was the most potent and gave a similar MPC value to that of the methoxy derivative (*R*)-**20p** (Table 1, entry 7).

Then we decided to evaluate the effect of the substitution pattern in fragment B by varying R_1 (hydrogen, methyl and ethyl) and including mono *ortho*, *meta* or *para* R_2-groups, while keeping the phenolic unit in fragment A. The compounds tested and their potencies towards *Tetrahymena* are compiled in Table 2.

Table 2. Activity of compounds **25** towards *Tetrahymena.*



Entry	Substance	R	R_2	MPC µg/ml (48 h)[a)]	MPC µg/ml (7 d)[a)]
1	**25a**	H	H	>64	>64
2	**25b**	H	*p*-F	>64	>64
3	**25c**	H	*m*-F	>64	>64
4	**25d**	H	*o*-F	>64	>64
5	(*R*)-**25e**	CH_3	H	8/16 [b)]	16/16 [b)]
6	(*R*)-**25f**	CH_3	*p*-F	32	32
7	(*R*)-**25g**	CH_3	*p*-CH_3	>64	>64
8	(*R*)-**25i**	CH_3	*p*-Br	8/8[b)]	8/8 [b)]
9	(*rac*)-**25j**	CH_3	*p*-CF_3	32	32
10	*(R)*-**25k**	CH_3	*o*-F	64	>64
11	*(Rac)*-**25l**	CH_3	*o*-CH_3	64	>64
12	*(R)*-**25m**	CH_3	*m*-F	16	16
13	*(Rac)*-**25n**	CH_3	*m*-CH_3	32	32
14	*(R)*-**25q**	Et	H	32	32
15	(*S*)-**25e**	CH_3	H	8/16 [b)]	8/16 [b)]
16	(*S*)-**25i**	CH_3	Br	8	16

[a)] The MPC values were determined by averaging three parallel measurements. [b)] Values given represent a second triplicate ground of testing.

The unsubstituted benzylamine derivative **25a** and three fluoro substituted benzylamine derivatives, **25b-d**, were all inactive in the concentration range tested (Table 2, entries 1-4). However, to our satisfaction (*R*)-**25e**, PKI-166,[43] having a *para*-hydroxyphenyl at C-6 and a 1-phenylethanamine substituent as C-4, proved to be potent (MPC: 8-16 µg/mL Entry 5),

indicating the importance of a chiral centre. Keeping the chiral R_1 group as methyl, and introducing rather conservative variations in the *para* position in terms of size and electronic properties, gave an MPC = 32 µg/mL for the fluoro derivative **25f**, while the methyl analogue **25g** was inactive. By changing the *para* substituent to bromo, (*R*)-**25i**, a MPC value of 8 µg/mL was obtained. Compared to the activity of the fluoro containing compound **25f** this indicates that a combination of both increased size and polarisability might be beneficial for achieving good potency. Also, the racemic trifluoromethyl derivative (*rac*)-**25j** showed activity (MPC = 32 µg/mL). *Ortho* and *meta* substitution by fluoro or a methyl substituent lowered the toxicity, but the *meta*-fluoro derivative **25m** showed appreciable protozoacidal activity (MPC: 16 µg/mL, entry 13). It was further investigated how the potency was affected by extending the chain length of R_1, but the result for **25q** containing a 1-phenylpropanamine substituent at C-4 (MPC = 32 µg/mL, entry 14) did not encourage further evaluation. To verify the importance of stereochemistry for the toxicity profile of these compounds we also synthesised and analysed for the effect of the (*S*)-enantiomers of the 1-phenylethanamine containing **25e** and its *para*-bromo substituted derivative **25i**. Both were found to be highly potent (Table 2, entries 15-16).

Some of the compounds evaluated in this study are efficient inhibitors of EGFR-TK *in vitro*.[37] These kinases depends on activation from the epidermal growth factor (EGF), which is a known signalling polypeptide in *Tetrahymena*.[44] However, we did not find any correlation between the *in vitro* activity towards EGFR-TK and the MPC values. This might indicate that receptors found in *Tetrahymena* are structurally different to the human version. To investigate if other kinases might be the target, the *para*-hydroxyphenyl derivative **25e** was evaluated against a panel of 124 kinases. Low inhibitory potency was observed in most cases (data not shown). Kinases which were inhibited to a degree of 25% or more at 50 nM are compiled in Figure 2. Of these kinases, protein kinase B,[45-47] calmodulin dependant enzymes,[48-49] protein kinase C,[9-50] and ERK1 type proteins,[51] are found in protozoa and represent possible sites of action for the pyrrolopyrimidines.

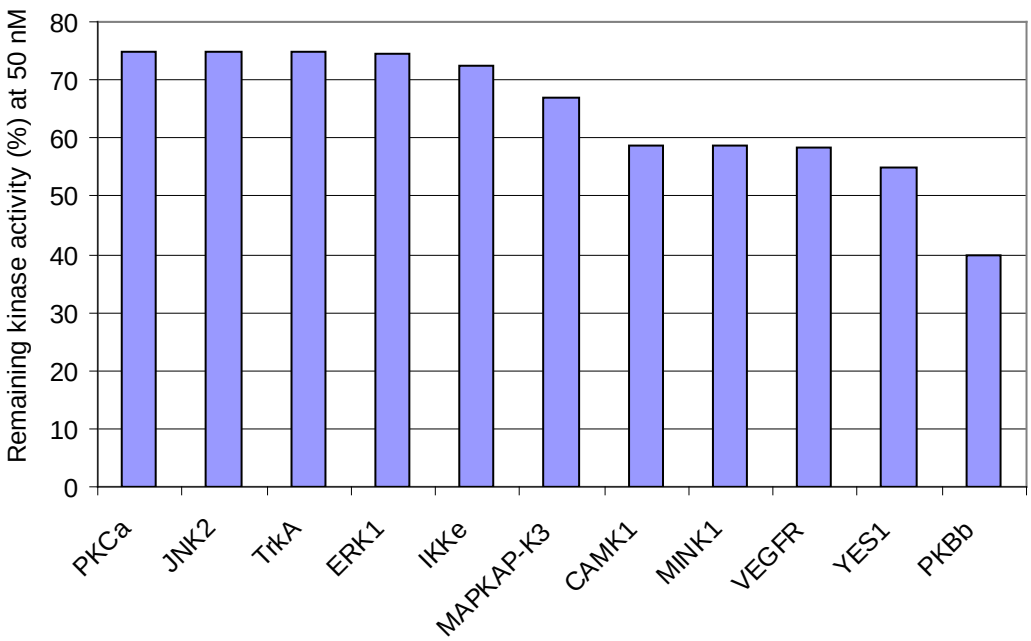


Figure 2. Effect of **25e** (50 nM) on the activity of a selection of kinases. PKCα: Protein kinase C alpha; JNK2: c-Jun N-terminal kinase/mitogen-activated protein kinase, TrkA: Neurotrophic tyrosine kinase receptor type 1, ERK1: extracellular-signal-regulated kinase; IKKe: inhibitory κB kinase, MAPKAP-K3, MAPK-activated protein kinase 3; CAMK 1: calmodulin-dependent kinase: MINK1: misshapen-like kinase 1 VEGFR: vascular endothelial growth factor receptor, Yes 1: Yamaguchi sarcoma viral oncogene homologue. PKBb: protein kinase B.

The toxicity/potency of the compounds presented in this study against *Tetrahymena* crucially depended on the presence of a *para* phenolic group in fragment A. This might be due to better solubility and bioavailability than for **20**-**24**, or that the hydroxyl function engages in critical bonding interaction. In *T. pyriformis* there is evidence for a transmembrane efflux pump as a detoxification mechanism, preferably excluding lipophilic compounds.[52] This might be an alternative explanation for the observed toxicity differences seen on introduction of the 4-hydroxyl group in fragment A. As *Tetrahymena* has only limited CYP-450 dependent metabolism activity,[53] it is less likely that the toxicity is due to oxidation of the *para*-hydroxyphenyl unit of **25** leading to aromatic 1,2-dienones, which are typical Michael acceptors in reaction with bio macromolecules.[16,18] The low level of CYP-450 enzymes should also exclude the possible formation of active compounds from putative precursors such as the C-6 phenyl substituted derivative **21**. Furthermore, the MPC values were affected by the substitution pattern and the presence of a chiral centre in fragment B. All the above indicates that there is a specific mode of action involving a defined 3-dimentional receptor target. However, the fact that both enantiomers showed toxicity is suggesting that the pyrrolopyrimidines could have multiple modes of action, or that the target is rather flexible.

As a model for unspecific toxicity the study shows that introduction of a 4-hydroxyl group in fragment A could be problematic for EGFR-TK inhibitors, but also that the toxicity profile could be modulated by the substitution pattern both in fragment A and B. Human toxicity issues have in fact been seen for **25e**.[54]

4. Conclusion

A series of pyrrolopyrimidines have been tested for their protozoacidal activity against *Tetrahymena*. Five compounds were found to be highly active (MPC 8-16 µg/mL). The identified compounds do not contain the typical groups which trigger non specific toxicity effects. The presence of a *para* phenolic group in position 6 (fragment A), and a chiral centre in the 4-benzylamine (fragment B) enhanced the potency considerably. *Ortho*-substitution in fragment B, and an electron donating methyl group in the *para* position reduced the toxicity. There is no evidence that EGFR-TK kinases are targets for these compounds in *Tetrahymena*, however, kinase profiling identified other potential sites of action. The detailed mechanism will be investigated in continuing work. The presented structure-activity relationships could be used as guidelines for targeting other, medically more important protozoa.

Acknowledgements

The group is thankful to the Anders Jahres foundation for financial support. Roger Aarvik is thanked for technical support. Susana Villa Gonzalez is acknowledged for HRMS experiments.

References

[1] M.J.G.Farthing, Treatment options for the eradication of intestinal protozoa, Nat.Clin.Pract.Gastroenterol.Hepatol. 3 (2006) 436-445.

[2] H.B.Fung, T.L.Doan, Tinidazole: a nitroimidazole antiprotozoal agent, Clin.Ther. 27 (2005) 1859-1884.

[3] J.A.Castro, M.Montalto de Mecca, L.C.Bartel, Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis), Hum.Exp.Toxicol. 25 (2006) 471-479.

[4] H.Cerecetto, M.Gonzalez, Synthetic medicinal chemistry in Chagas' disease: compounds at the final stage of "hit-to-lead" phase, Pharmaceuticals 3 (2010) 810-838.

[5] A.Klokouzas, S.Shahi, S.B.Hladky, M.A.Barrand, H.W.van Veen, ABC transporters and drug resistance in parasitic Protozoa, Int.J.Antimicrob.Agents 22 (2003) 301-317.

[6] S.M.Townson, P.F.L.Boreham, P.Upcroft, J.A.Upcroft, Resistance to the nitroheterocyclic drugs, Acta Trop. 56 (1994) 173-194.

[7] M.Matsushita, K.D.Janda, Histidine kinases as targets for new antimicrobial agents, Bioorg.Med.Chem. 10 (2002) 855-867.

[8] M.Parsons, J.A.Ledbetter, G.L.Schieven, Inhibitors of tyrosine phosphorylation in protozoa as drugs , EP 507256, (1992).

[9] F.J.Gamo, L.M.Sanz, J.Vidal, C.de Cozar, E.Alvarez, J.L.Lavandera, D.E.Vanderwall, D.V.S.Green, V.Kumar, S.Hasan, J.R.Brown, C.E.Peishoff, L.R.Cardon, J.F.Garcia-Bustos, Thousands of chemical starting points for antimalarial lead identification, Nature 465 (2010) 305-310.

[10] R.Diaz-Gonzalez, F.M.Kuhlmann, C.Galan-Rodriguez, L.Madeira da Silva, M.Saldivia, C.E.Karver, A.Rodriguez, S.M.Beverley, M.Navarro, M.P.Pollastri, The susceptibility of trypanosomatid pathogens to PI3/mTOR kinase inhibitors affords a new opportunity for drug repurposing, PLoS Neglected Trop.Dis. 5 (2011) e1297.

[11] K.Mensa-Wilmot, Tyrosine kinase inhibitors as anti-kinetolastid and anti-apicomplexan protozoal agents , WO 2008066755, (2007).

[12] M.P.Sauvant, D.Pepin, E.Piccinni, Tetrahymena pyriformis: a tool for toxicological studies. A review, Chemosphere 38 (1999) 1631-1669.

[13] M.Hewitt, M.T.D.Cronin, P.H.Rowe, T.W.Schultz, Repeatability analysis of the Tetrahymena pyriformis population growth impairment assay, SAR QSAR Environ.Res. 22 (2011) 621-637.

[14] T.W.Schultz, C.L.Sparfkin, A.O.Aptula, Reactivity-based toxicity modelling of five-membered heterocyclic compounds: Application to Tetrahymena pyriformis, SAR QSAR Environ.Res. 21 (2010) 681-691.

[15] S.J.Enoch, M.T.D.Cronin, T.W.Schultz, J.C.Madden, An evaluation of global QSAR models for the prediction of the toxicity of phenols to Tetrahymena pyriformis, Chemosphere 71 (2008) 1225-1232.

[16] F.Bajot, M.T.D.Cronin, D.W.Roberts, T.W.Schultz, Reactivity and aquatic toxicity of aromatic compounds transformable to quinone-type Michael acceptors, SAR QSAR Environ.Res. 22 (2011) 51-65.

[17] T.I.Netzeva, T.W.Schultz, QSARs for the aquatic toxicity of aromatic aldehydes from Tetrahymena data, Chemosphere 61 (2005) 1632-1643.

[18] A.O.Aptula, D.W.Roberts, M.T.D.Cronin, T.W.Schultz, Chemistry-Toxicity Relationships for the Effects of Di- and Trihydroxybenzenes to Tetrahymena pyriformis, Chem.Res.Toxicol. 18 (2005) 844-854.

[19] T.W.Schultz, G.D.Sinks, M.T.D.Cronin, Quinone-induced toxicity to Tetrahymena: structure-activity relationships, Aquat.Toxicol. 39 (1997) 267-278.

[20] M.Koubar, M.H.Rodier, R.A.Garduno, J.Frere, Passage through Tetrahymena tropicalis enhances the resistance to stress and the infectivity of Legionella pneumophila, FEMS Microbiol.Lett. 325 (2011) 10-15.

[21] W.K.Whitekettle, Control of protozoa and protozoan cysts that harbor Legionella using quaternary ammonium salts , US 20050027010, (2005).

[22] P.J.Jakobsen, O.Enger, Treatment of parasite diseases using vitamin K3 , WO 2009063044, (2009).

[23] M.P.Leibowitz, J.K.Chettri, R.Ofir, D.Zilberg, Treatment development for systemic Tetrahymena sp. infection in guppies, Poecilia reticulata peters, J.Fish Dis. 33 (2010) 473-480.

[24] Y.Muto, Y.Tanabe, K.Kawai, Y.Okano, H.Iio, Climacostol inhibits Tetrahymena motility and mitochondrial respiration, Cent.Eur.J.Biol. 6 (2011) 99-104.

[25] H.Hegyesi, P.Kovacs, G.Csaba, Chloroquine inhibits the insulin binding and the imprinting of nuclear envelope in Tetrahymena, Acta Microbiol.Hung. 39 (1992) 289-293.

[26] J.R.Nilsson, Effects of chloramphenicol on the physiology and fine structure of Tetrahymena pyriformis GL: correlation between diminishing inner mitochondrial membrane and cell doubling, Protoplasma 135 (1986) 1-11.

[27] C.Wu, P.Clift, C.H.Fry, J.A.Henry, Membrane action of chloramphenicol measured by protozoan motility inhibition, Arch.Toxicol. 70 (1996) 850-853.

[28] A.Kaczanowski, M.Ramel, J.Kaczanowska, D.Wheatley, Macronuclear differentiation in conjugating pairs of Tetrahymena treated with the antitubulin drug nocodazole, Exp.Cell Res. 195 (1991) 330-337.

[29] G.Csaba, P.Kovacs, E.Pallinger, Presence and localization of epidermal growth factor (EGF)- and EGF-receptor-like immunoreactivity in Tetrahymena, Cell Biol.Int. 28 (2004) 491-496.

[30] L.Chiu, Pharmaceutical compositions containing protein kinase B inhibitors and epidermal growth factor receptor tyrosine kinase inhibitors for treating cancer , CN 101653606, (2008).

[31] F.Chen, V.Leick, The protozoan Tetrahymena as a bioindicator to screen bioactive substances, J.Microbiol.Methods 59 (2004) 233-241.

[32] P.Kovacs, G.Csaba, Effect of inhibitors and activators of tyrosine kinase on insulin imprinting in Tetrahymena, Cell Biochem.Funct. 10 (1992) 267-271.

[33] F.Hans, S.Dimitrov, Histone H3 phosphorylation and cell division, Oncogene 20 (2001) 3021-3027.

[34] W.Li, S.Zhang, O.Numata, Y.Nozawa, S.Wang, TpMRK regulates cell division of Tetrahymena in response to oxidative stress, Cell Biochem.Funct. 27 (2009) 364-369.

[35] S.Nakashima, S.Wang, N.Hisamoto, H.Sakai, M.Andoh, K.Matsumoto, Y.Nozawa, Molecular cloning and expression of a stress-responsive mitogen-activated protein kinase-related kinase from Tetrahymena cells, J.Biol.Chem. 274 (1999) 9976-9983.

[36] J.Bartholomew, J.Reichart, R.Mundy, J.Recktenwald, S.Keyser, M.Riddle, H.Kuruvilla, GTP avoidance in Tetrahymena thermophila requires tyrosine kinase activity, intracellular calcium, NOS, and guanylyl cyclase, Purinergic Signalling 4 (2008) 171-181.

[37] S.J.Kaspersen, C.Sørum, V.Willassen, E.Fuglseth, E.Kjøbli, G.Bjørkøy, E.Sundby, B.H.Hoff, Synthesis and *in vitro* EGFR (ErbB1) tyrosine kinase inhibitory activity of 4-*N*-substituted 6-aryl-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-amines, Eur.J.Med.Chem. 46 (2011) 6002-6014.

[38] E.Otterholt, C.Charnock, Identification and phylogeny of the small eukaryote population of raw and drinking waters, Water Res. 45 (2011) 2527-2538.

[39] J.Bain, L.Plater, M.Elliott, N.Shpiro, C.J.Hastie, H.McLauchlan, I.Klevernic, J.S.Arthur, D.R.Alessi, P.Cohen, The selectivity of protein kinase inhibitors: a further update, Biochem.J. 408 (2007) 297-315.

[40] C.Sørum, N.Simic, E.Sundby, B.H.Hoff, 1H,13C and 19F NMR data of N-substituted 6-(4-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amines in DMSO-d6, Magn.Reson.Chem. 48 (2010) 244-248.

[41] X.Cai, C.Qian, S.Gould, Fused bicyclic pyrimidines as PTK inhibitors containing a zinc binding moiety , WO 2008033745, (2007).

[42] E.Toja, A.DePaoli, G.Tuan, J.Kettenring, Synthesis of 2-amino-3-(ethoxycarbonyl)pyrroles, Synthesis (1987) 272-274.

[43] G.Caravatti, J.Bruggen, E.Buchdunger, R.Cozens, P.Furet, N.Lydon, T.O'Reilly, P.Traxler, Pyrrolo[2,3-d]pyrimidine and pyrazolo[3,4-d]pyrimidine derivatives as selective inhibitors of the EGF receptor tyrosine kinase, ACS Symposium Series 796 (2001) 231-244.

[44] I.V.Schemarova, G.V.Selivanova, T.D.Vlasova, The influence of the EGF on proliferative signal transduction in ciliate Tetrahymena pyriformis, Tsitologiya 49 (2007) 156-160.

[45] A.Vaid, P.Sharma, PfPKB, a Protein Kinase B-like Enzyme from Plasmodium falciparum: II. Identification of Calcium/Calmodulin as Its Upstream Activator and Dissection of a Novel Signaling Pathway, J.Biol.Chem. 281 (2006) 27126-27133.

[46] J.Guergnon, F.Dessauge, F.Traincard, X.Cayla, A.Rebollo, P.E.Bost, G.Langsley, A.Garcia, A PKA survival pathway inhibited by DPT-PKI, a new specific cell permeable PKA inhibitor, is induced by T. annulata in parasitized B-lymphocytes, Apoptosis 11 (2006) 1263-1273.

[47] V.Pascuccelli, C.Labriola, M.T.Tellez-Ino, A.J.Parodi, Molecular and biochemical characterization of a protein kinase B from Trypanosoma cruzi, Mol.Biochem.Parasitol. 102 (1999) 21-33.

[48] K.Gonda, M.Katoh, K.Hanyu, Y.Watanabe, O.Numata, Ca2+/calmodulin and p85 cooperatively regulate an initiation of cytokinesis in Tetrahymena, J.Cell Sci. 112 (1999) 3619-3626.

[49] J.Hirano-Ohnishi, Y.Watanabe, Calcium/calmodulin-dependent phosphorylation of ciliary β-tubulin in Tetrahymena, J.Biochem. 105 (1989) 858-860.

[50] D.L.Hassenzahl, N.K.Yorgey, M.D.Keedy, A.R.Price, J.A.Hall, C.C.Myzcka, H.G.Kuruvilla, Chemorepellent signaling through the PACAP/lysozyme receptor is mediated through cAMP and PKC in Tetrahymena thermophila, J.Comp.Physiol., A 187 (2001) 171-176.

[51] M.Arslanyolu, Cloning and partial characterization of Tetrahymena thermophila Mitogen activated protein kinase 3 (TtMPK3) gene, J.Appl.Biol.Sci. 1 (2007) 1-11.

[52] M.Bamdad, P.Brousseau, F.Denizeau, Identification of a multidrug resistance-like system in Tetrahymena pyriformis: evidence for a new detoxication mechanism in freshwater ciliates, FEBS Lett. 456 (1999) 389-393.

[53] H.Iida, J.Kimura, J.J.Johnson, L.J.Marnett, Microsomal drug hydroxylase activity of Tetrahymena pyriformis, Comp.Biochem.Physiol.C 63C (1979) 381-387.

[54] T.Takada, H.M.Weiss, O.Kretz, G.Gross, Y.Sugiyama, Hepatic transport of PKI166, an epidermal growth factor receptor kinase inhibitor of the pyrrolo-pyrimidine class, and its main metabolite, ACU154, Drug Metab.Dispos. 32 (2004) 1272-1278.