Bioorganic & Medicinal Chemistry Letters 20 (2010) 3486–3490

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Towards Gram-positive antivirulence drugs: New inhibitors of *Streptococcus agalactiae* Stk1

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ARTICLE INFO

Article history:
Received 3 March 2010
Revised 3 May 2010
Accepted 3 May 2010
Available online 7 May 2010

Keywords:
Antivirulence drugs
Antibacterial
STK
Stk1
Streptococcus agalactiae
Gram-positive
Serine/threonine kinase

ABSTRACT

A structure–activity relationship study from a screening hit and structure-based design strategy has led to the identification of bisarylureas as potent inhibitors of *Streptococcus agalactiae* Stk1. As this target has been directly linked to bacterial virulence, these inhibitors can be considered as a promising step towards antivirulence drugs.

Bacterial resistance to antibiotics is a serious public health problem which increasingly limits the effectiveness of current drugs.[1–3] *Streptococcus agalactiae* (Group B Streptococci, GBS), a Gram-positive pathogen, represents the major cause of infection during the neonatal period leading to pneumonia, sepsis and meningitis. To fulfill the urgent medical and social need of effective antibacterial therapies, our approach focuses on developing new molecules with innovative mechanisms of action.

Kinase-mediated phosphorylation of intracellular proteins is a crucial pathway by which signals are transmitted within the cell. In bacteria, communication is achieved by two-component systems (TCS) as well as by eukaryotic-type serine/threonine kinases (STK).[4–6] In the past few years a conserved family of bacterial STK, unique to Gram-positive species, has emerged as an attractive potential antibacterial target.[7–13] These kinases are proposed to be regulators of metabolic processes, including transcription, growth, differentiation, cell development, sporulation, biofilm formation and interaction with host cells. Recently, direct relationships between STKs and bacterial virulence have also been established.[7a,11–14] For instance, mutations or deletion of the *Stk1* gene of *S. agalactiae* exhibited pleiotropic effects on growth, cell segregation and effectiveness in murine models of virulence.[8,9,15] We report herein our approach to the identification of *S. agalactiae* Stk1 inhibitors and the preliminary structure–activity relationship (SAR) generated from a family of 2-aryl-purines.

In *S. agalactiae,* Stk1 is a transmembrane protein harboring a serine/threonine kinase domaine located in the cytoplasm of the bacteria and 3 PASTA (for 'penicillin-binding protein and serine/threonine kinase associated') domains outside the bacteria.[8] The soluble kinase domain has been expressed in *Escherichia coli* with an N-Terminal 6-His tag.[16] Stk1 can phosphorylate both serine and threonine residues, and recognizes myelin basic protein (MBP) as surrogate substrate which has been chosen in our assays.[8a,17] High-throughput screening of our in-house library of molecules allowed identification of the 6-(3-aminopropanol)-substituted purine[18] compound **1b** as an inhibitor of *S. agalactiae* Stk1 with an IC_{50} of 66 μM. In order to better understand the potential mode of binding of that molecule, a model of the ATP-binding site was built up with Easypred[19] by homology modelling based on the sequence and available structure of the *Mycobacterium tuberculosis* STK enzyme PknB (pdb code: 1O6Y).[20] *M. tuberculosis* H37rv STK shares 32/52% of identity/similarity with the *S. agalactiae* NEM316 Stk1. Analogues were also synthesized as outlined in Scheme 1 to investigate the SAR of this series.

The 3-aminopropanol was introduced on C6 position of the 2,6-dichlopurine by refluxing in butanol to afford compound **1a** with 72% yield. Alkylation led exclusively to substituted compounds on position N9 (**1b–d**).[21] Finally, palladium-mediated reactions of

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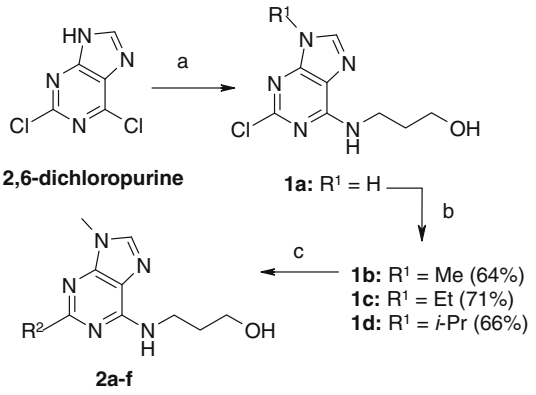
doi:10.1016/j.bmcl.2010.05.004



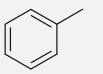
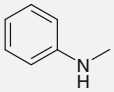
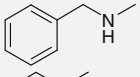
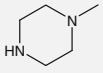
Scheme 1. Reagents and conditions: (a) 3-aminopropan-1-ol, TEA, *n*-BuOH, 120 °C, overnight, 72%; (b) NaH (60% in oil), alkyl halide, DMF, rt, overnight; (c) boronic acid, K_2CO_3, $Pd(PPh_3)_4$, toluene, 100 °C, overnight, 19–43%; or aniline, *t*-BuONa, Pd_2dba_3, R(+)-BINAP, toluene, 80 °C, overnight, 11%; or amine, neat, reflux, 2.5–5 h, 12–86%.

the C2 chlorine with boronic acids or aniline provided derivatives **2a–c** while amino-derivatives **2d–f** were obtained by refluxing in neat amines.

The methyl group at purine N9 position was rapidly determined as optimal for the activity as seen in Table 1, since modifications by larger alkyl substituents or in contrast no substitution resulted in a significant decrease of activity. Exploration of the position C2 indicated a hydrophobic aromatic bias. Indeed, a phenyl group in compound **2a** is the most promising substitution with a fivefold improvement of activity compared to the initial hit **1b**. In contrast, the more hydrophilic pyridyl analogue **2b** was less active than **2a**. The lipophilic aniline and benzylamine derivatives **2c** and **2d** were more active than **1b**, although 2–4 times less potent than **2a**, whereas the morpholino derivative **2e** and the charged piperazine **2f** were notably less active than **2a**.

All these observations were in complete agreement with the binding mode of **2a** (Fig. 1) obtained by docking in the ATP site of our model using QXP/Flo+.[22] The 9-methyl purine ring system fits well into the canonical ATP-binding site, the heterocycle being sandwiched between the lipophilic residues Ala 40 and Leu 143. Two crucial hydrogen bonding interactions occurring between the nitrogen N7 of the purine and the nitrogen of the propanol-

Table 1
S. agalactiae Stk1 inhibitory activities of compounds **1** and **2**

Compound	R^1	IC_{50}[a] (μM)	Compound	R^2	IC_{50}[a] (μM)
1a	H	>300	**2a**		12
1b	Me	66	**2b**		34
1c	Et	175	**2c**		26
1d	*i*Pr	>300	**2d**		48
			2e		66
			2f		110

[a] Values are means of three experiments.

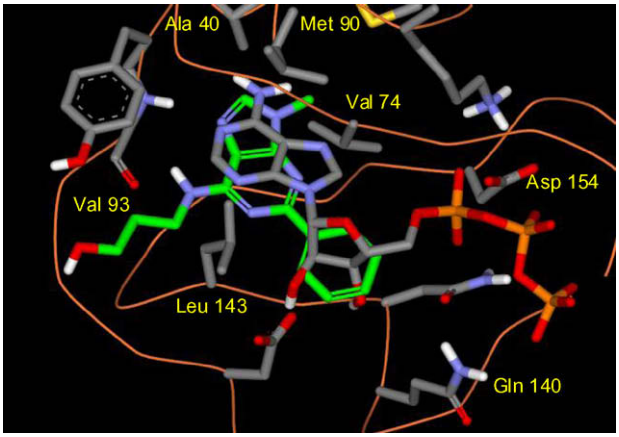
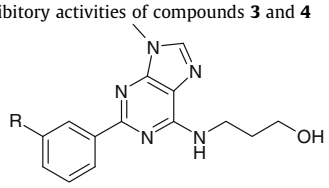
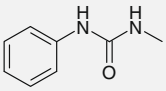
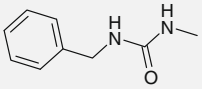
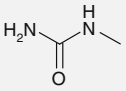
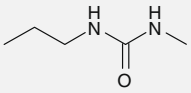
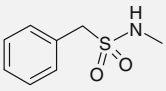
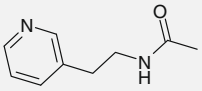
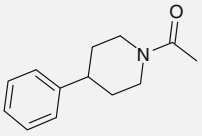
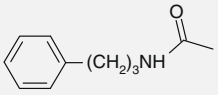


Figure 1. Overlay of **2a** (green) with ATP in the active site of *S. agalactiae* Stk1 with expanded key residues.

Table 2
S. agalactiae Stk1 inhibitory activities of compounds **3** and **4**

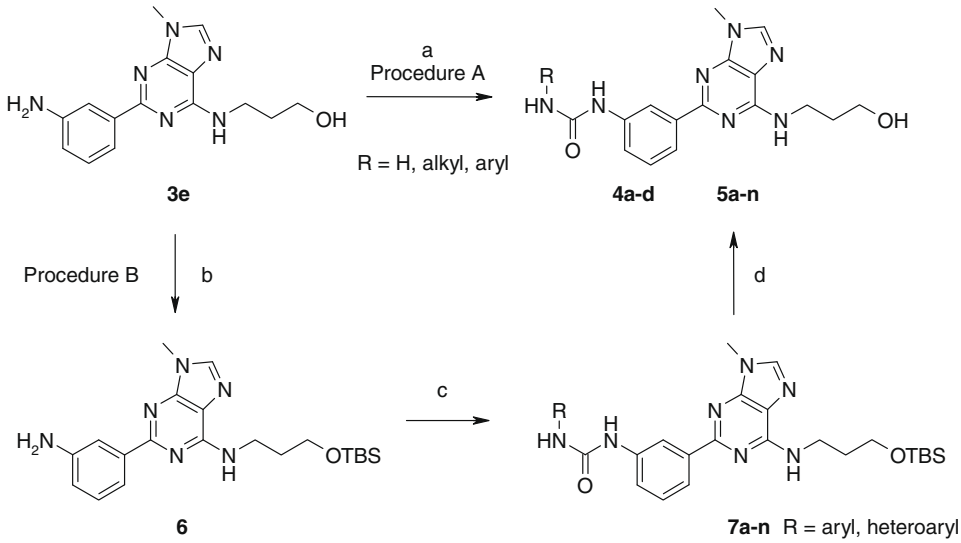


3a-h, 4a-h

#	R	IC_{50}[a] (μM)	#	R	IC_{50}[a] (μM)
3a	Cl	3	**4a**		1
3b	NO_2	3	**4b**		7.5
3c	CF_3	4	**4c**		12
3d	Ph	4	**4d**		18
3e	NH_2	13	**4e**		22
3f	$NHSO_2Me$	15	**4f**		38
3g	NHAc	18	**4g**		174
3h	CO_2H	39	**4h**		>300

[a] Values are means of three experiments.

amine side-chain of the compounds with, respectively, the NH and CO of Val 93 in the hinge region of the protein complete the binding. In addition, similarly to the known CDK inhibitors such as olomoucine,[23] compound **2a** made a close lipophilic contact with its methyl at position N9 in the deeply buried hydrophobic

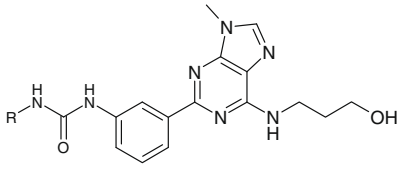


Scheme 2. Reagents and conditions: procedure A: (a) R = H: urea (4 equiv), water, AcOH (1.3 equiv), HCl, reflux, 4%; R = alkyl, aryl: RNCO (0.8–4.6 equiv), $NaHCO_3$ (0–6 equiv), toluene or DCE or THF, rt to reflux, 20–120 h, 4–27%; procedure B: (b) TEA (2 equiv), TBDMSCl (1.5 equiv), DMF, rt, 5 h, 85%; (c) **6**, 4-nitrophenylchloroformate (1.2 equiv), THF, rt, 45 min–2 h, then RNH_2 (1.5 equiv), TEA (2 equiv), 50 °C, 0.5–5 h; (d) TBAF (3–5 equiv), THF or ammonium fluoride (13 equiv), MeOH, rt to reflux, 20 h, 40–46% over two steps.

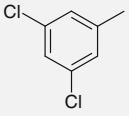
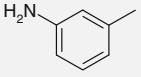
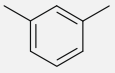
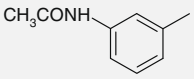
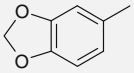
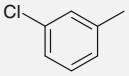
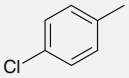
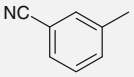
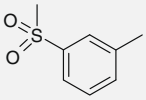
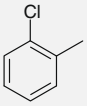
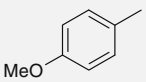
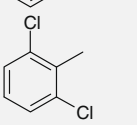
pocket lined by Met 90, Val 74 and the methyl of Thr 153. It appears also clearly that there is little room for bulkier substituents.

According to this model of docking we hypothesized that growing the molecule from the *meta* position of the phenyl ring towards the

Table 3
S. agalactiae Stk1 inhibitory activities of compounds **5**



5a-n

#	R	IC_{50}[a] (μM)	#	R	IC_{50}[a] (μM)
5a[b]		0.16	**5h**[c]		0.73
5b[b]		0.2	**5i**[c]		0.93
5c[b]		0.28	**5j**[c]		0.96
5d[b]		0.32	**5k**[b]		1.6
5e[b]		0.49	**5l**[b]		2
5f[c]		0.64	**5m**[b]		16
5g[b]		0.63	**5n**[b]		59

[a] Values are means of three experiments.
[b] Synthesis according to Scheme 2, procedure A.
[c] Synthesis according to Scheme 2, procedure B.

phosphate region of the ATP site could generate additional interactions, especially with the hydrophilic residues Gln140 or Asp154 and the remote lipophilic loop between Gly 21 and Ala 24.

A series of substitution was therefore introduced at the position *meta* of the aryl group by Suzuki couplings with the corresponding boronic acids to provide compounds **3a–h** (Table 2). With the exception of substituents Cl, CF_3, NO_2 and Ph of **3a–d**, no improvement was achieved with short amino, sulfonylamido, amido or carboxylic acid substitutions (compounds **3e–h**). The biochemical characterization of **3c** assessed that this compound worked by competitive inhibition with regard to ATP, in agreement with our model.[24] At this stage, some counter-screening on the related eukaryotic protein kinase A was performed on a few compounds. The results indicated a potential for selectivity of this series with respect to eukaryotic kinases.[25]

The next step aimed at extending the compound. Reaction with isocyanates afforded urea analogues **4a–d** (Scheme 2, procedure A), while longer sulfonamido and amido groups were obtained by standard methods (sulfonyl chloride, 4-DMAP, pyridine and amine, HATU, EDCI, DCM, respectively). A clear gain of activity was obtained with the phenylurea **4a** at 1 μM. Interestingly the mono-aryl ureas **4b–d** were significantly less potent, highlighting the importance of the secondary aryl group for activity. The benzylsulfonamide **4e** turned out less active than the corresponding benzylurea **4b**. Finally, we observed that longer and more flexible substitutions yielded weaker activities as illustrated by compounds **4f–g** or complete inactivity in the case of **4h**.

The encouraging result obtained with **4a** prompted us to further explore this urea series. Indeed, the modelling and docking of **4a** into the ATP cavity indicated that the gain of potency could be explained by two new hydrogen bonds with the targeted residues Gln140 or Asp154 as well as a favourable hydrophobic contact with the Gly 21-Ala 24 loop. We also observed that the position *meta* of the second phenyl group pointed into the direction of Ileu 157 and if properly substituted could therefore generate additional lipophilic contact. New bis-aryl ureas have been synthesized either via commercially available isocyanates (Scheme 2, procedure A) or via activation of the aniline moiety of **3e** as a 4-nitrophenylcarbamate (Scheme 2, procedure B). According to the model, we observed that the position *ortho* (**5m–n**) was clearly less favourable for substitution than the *para* or *meta* ones (Table 3). This was also consistent with the requirement of planarity of the aryl-urea-aryl system to interact with the abovementioned key residues and to fit in the cavity below the loop Gly 21-Ala 24. In agreement with our model, the most interesting compounds **5a–d** were substituted by lipophilic substituents at the *meta* position such as *m*,*m*′-dichloro, methyl, piperonyl and chloro (Fig. 2) in contrast to the *para*-chloro derivative **5k** which was fivefold less active than its *meta* counterpart **5d**. The more polar groups such as amino, acetamido, cyano or methylsulfonyl (**5e,f,h,i**), while being more active than **4a** were less potent than **5a–d**. As shown by **5j** and **5l**, the replacement of the phenyl group by a heteroarene (pyridine or thiophene) was not deleterious for the activity when compared to **4a**.

Finally, having identified **5d** as a potent inhibitor, we decided to come back to the propanol moiety and explore its importance for the activity. A more convergent pathway was set up (Scheme 3) starting from 2,6-dichloropurine via a common methylsulfonyl intermediate **10** that was treated with amines to afford the desired compounds **11a–f** in moderate yields.

In agreement with the proposed hydrogen-bond donation of the propanolamine nitrogen with the protein, compound **11f** was inactive. Although we suspected from the docking model a potential hydrogen bond of the hydroxy group of the propanol with Tyr 92, the activity of derivatives **11a–c** clearly demonstrated that this interaction did not occur. The propanolamine side-chain was most likely making hydrophobic contact with the backbone of the protein as shown by the good activity of **11a–d** compared to the unsubstituted compound **11e** (Table 4).

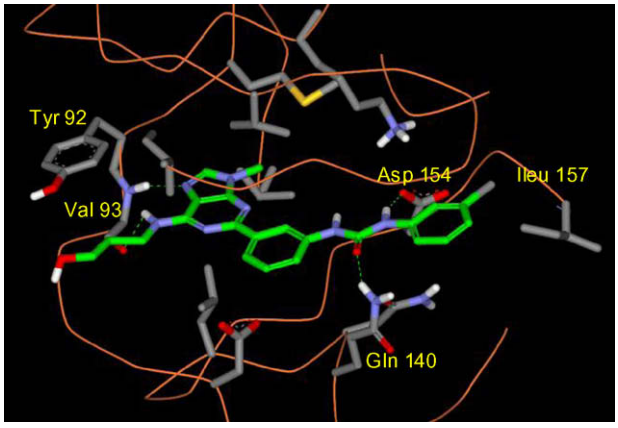
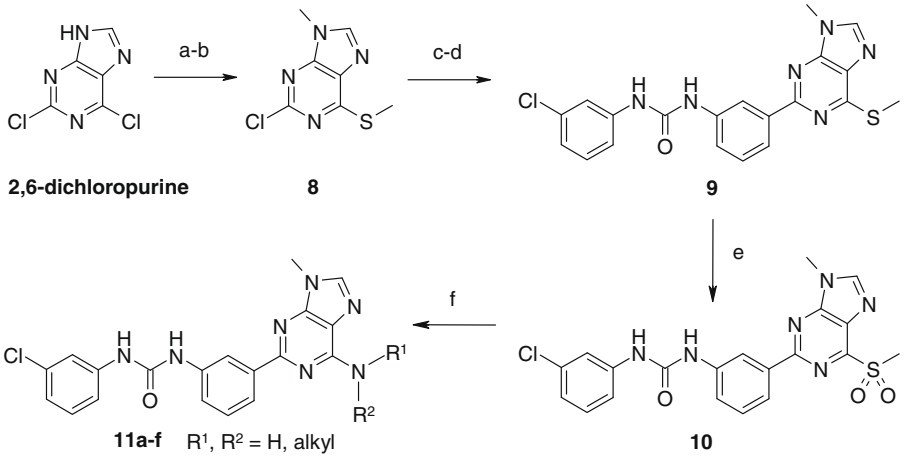
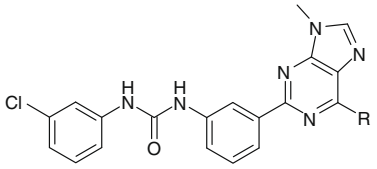


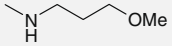
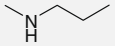
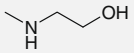
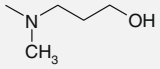
Figure 2. Compound **5d** (green) in the active site of *S. agalactiae* STK with expanded key residues.



Scheme 3. Reagents and conditions: (a) K_2CO_3 (1.1 equiv), MeI (1.4 equiv), DMSO, rt, 2 h, 61%; (b) NaSMe (1.2 equiv), DMSO, rt, 2 h, 51%; (c) 3-aminophenyl boronic acid monohydrate (1.4 equiv), 2-cyclohexylphosphino-2′,6′-dimethoxybiphenyl (0.02 equiv), $Pd(OAc)_2$ (4 × 0.04 equiv), CH_3CN, reflux, 48 h, 21%; (d) 3-chlorophenyl isocyanate (0.8 equiv), THF, rt, 2 h, 64%; (e) *m*-CPBA (8 equiv), THF, rt, 23 h, 94%; (f) HNR^1R^2 (5 equiv), THF, reflux, 5 h, 14–50%.

Table 4
S. agalactiae Stk1 inhibitory activities of compounds **11**



#	R	IC_{50}[a] (μM)	#	R	IC_{50}[a] (μM)
11a		0.28	**11d**		0.48
11b		0.3	**11e**		1.3
11c		0.32	**11f**		>300

[a] Values are means of three experiments.

In conclusion, we have identified new bisarylurea derivatives as *S. agalactiae* Stk1 inhibitors in the course of a SAR study assisted by molecular modelling. These small molecules represent encouraging leads for the development of novel anti-infective drugs based on the concept of antivirulence.

Acknowledgment

The authors wish to thank Oséo for financial support (Grant A 06 05 033Q).

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16. In *S. agalactiae* GBS NEM316 strain, the relevant gene is gbs0307 with NP_734776 as the corresponding protein accession number.
17. Stk1 biochemical assays were based either on luminescent ATP detection, or on fluorescent ADP detection. They used the surrogate substrate MBP (myelin basic protein). The steady-state kinetic parameters of Stk1 were K_M(ATP) = 0.4 μM at MBP = 2 μM and K_M(MBP) = 0.06 μM at ATP=5 μM. In the luminescent assay, inhibitor in 3 μL dimethylsulfoxide (DMSO) was pre-incubated in a white polystyrene 96-well plate at room temperature for 30 min with 27 μL Stk1 in assay buffer AB (50 mM Hepes pH7.5, 0.5 mM $MnCl_2$, 0.012% Triton-X100, 1 mM DTT). 30 μL of MBP/ATP mix in AB were then added to start the reaction with final concentrations of 5 nM Stk1, 0.3 μM myelin basic protein (Sigma) and 0.3 μM ATP (Sigma). After 90 min of incubation at room temperature, 30 μL of the revelation mix were added: 2 nM luciferase (Sigma), 30 μM D-luciferin (Sigma), 100 μM *N*-acetylcysteamine (Aldrich). Luminescence intensity was immediately measured on an Analyst-HT (Molecular Devices) and converted into inhibition percentages. For IC_{50} determinations, the inhibitor was tested at 6–10 different concentrations, and the related inhibitions were fitted to a classical Langmuir equilibrium model using XLFIT (IDBS). In these conditions, the IC_{50} of the non specific kinase inhibitor staurosporin was 21 nM. In the fluorescent assay, the following components were pre-incubated for 30 min at room temperature in a black polystyrene 96-well plate: 5 μL inhibitor dissolved in DMSO and 45 μL Stk1 in AB. 50 μL of substrates-revelation mix in AB were then added in each well leading to the following final composition: 10 nM Stk1, 2 μM MBP, 0.3 μM ATP, 5 u/mL Pyruvate Kinase (Sigma), 50 μM phosphoenolpyruvate (Sigma), 5 u/mL Lactate des-hydrogenase (Sigma) and 3 μM NADH (Sigma). Fluorescence intensity of NADH (λ_{ex} = 360 nm, λ_{em} = 520 nm) was immediately measured kinetically on a Fluostar Optima (BMG). Inhibition percentages were derived from fitted initial velocities using XLFIT (IDBS).
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24. Compound **3c** (0–300 μM) was tested on Stk in the fluorescent assay (see note[20]) in the presence of 2 μM MBP and ATP (0.08–10 μM). NADH was raised at 5 μM. Initial velocities were fitted with XLFIT (IDBS) leading to intersecting lines on *y*-axis at $1/V_{max}$ in the double reciprocal plot (1/initial velocity versus 1/[ATP]). The K_i of **3c** was 2.9 μM.
25. Human protein kinase A (PKA) shares 99/99% of identity/similarity with its bovine counterpart. Both PKAs share 24/48% of identity/similarity with the *S. agalactiae* NEM316 Stk1. Screening **2a** and **3c** on the bovine PKA led to IC_{50}s >100 μM.