Development of Rubidium Flux Assay & HTS Campaign for Modulators of a Cation-Chloride Co-transporter

Yang Wen[1], Doris Roth[1], Thilo Enderle[1], Sikander Gill[2], Rajwant Gill[2], Sophia Liang[2], Dong Liang[2]

Roche Innovation Center Basel, Hoffmann-La Roche, Grenzacherstrasse 124, 4070 Basel, Switzerland[1]

Aurora Biomed Inc, 1001 E Pender Street Vancouver, BC, V6A 1W2, Canada[2]

I. Abstract

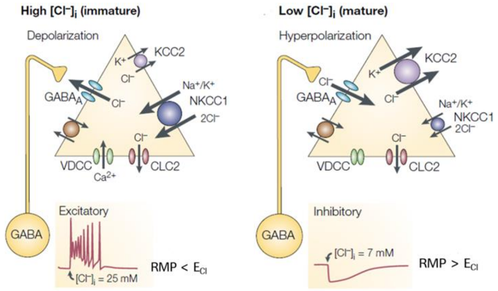
GABA developmental switch controls the intracellular chloride concentration and shifts the functional role of GABA between neonatal and adult stages from excitatory to inhibitory. The switch is mediated by the change in expression of two major ion co-transporters $Na^+K^+2Cl^-$ co-transporter (NKCC1) and K^+Cl^- co-transporter (KCC2). Alterations in this mechanism may result in CNS disorders such as autism spectrum disorder, epilepsy, and schizophrenia. Ion flux detection is the consensus approach to measure electro-neutral CCCs activity. While several approaches are available such as radioactive flux and fluorescence based techniques including Thalium Flux, atomic absorption spectrometry based rubidium (Rb^+) uptake assay was found to be the most optimal due to its specificity for measurement of the co-transporter activity. Here we describe the development of a high throughput rubidium uptake assay for measuring NKCC1 function, and the HTS campaign of 1.2 million compounds using Rb^+ uptake detection by AAS technology (ICR12000™, Aurora Biomed, Vancouver, Canada) to identify modulators of NKCC1.

II. Introduction

Development of hyperpolarizing effects of GABA requires a decrease of intraneuronal [Cl^-] due to a change in the ratio of two ion transporters with opposing functions (NKCC1 and KCC2). Defects in this switch can disrupt the normal balance of excitation and inhibition, which is a central hypothesis for the etiology of autism. It was found that NKCC1 inhibitor Bumetanide improved symptoms in autistic children and seizure frequency in epilepsy patients.

NKCC1 co-transports Na^+, K^+ and Cl^- at 1 : 1 : 2 stoichiometry. It's a non-electrogenic transporter. Manual and automated electrophysiology measurements are not directly amenable (due to electro-neutral NKCC1). Chloride sensor eYFP only offer low fluorescence with a 20 mM [Cl^-] change.

Ion flux detection by substitution of K^+ (152pm) with similar size cations Tl^+ (164pm) or Rb^+ (166pm), offers robust detection window. The Thallium assay is not a preferred option due to the generation of toxic waste. Therefore, the non-radioactive Rb^+ uptake assay was chosen to measure NKCC1 activity. A HTS campaign was planned in order to develop an NKCC1 inhibitor with high brain penetration and selectivity to maximize activity in the CNS.



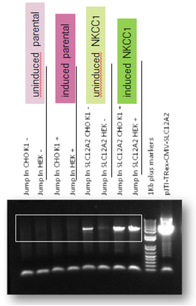
III. Objectives

- Develop & optimize an HTS assay for NKCC1 drug target
- Screen compound libraries against NKCC1 target

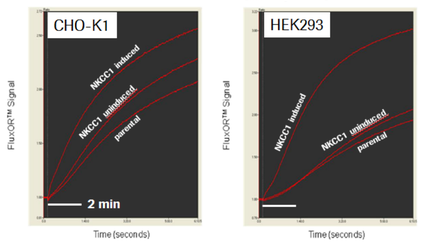
IV. Results & Discussion

Cell Line Generation:
Inducible NKCC1 cell line was generated by LifeTechnologies and validated by functional test (Tl^+-uptake)

Expression test (CHO, HEK)



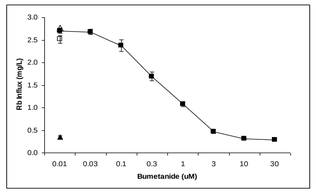
Functional test (Tl^+-uptake)



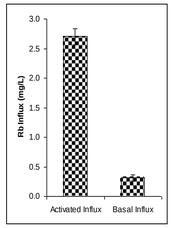
- HEK293 preferred over CHO-K1
- Measure uptake within first 2-3 minutes
- Background in parental cell lines
- Uptake sensitive to bumetanide (not shown here)

Assay Development (96 well):

Rb-uptake detection by AAS was developed by Aurora Biomed. Readings compared on both on ICR8000 and ICR12000.



Dose response curve of bumetanide on NKCC1 Rb^+ influx activated for 2 min (■). Positive control 30uM of bumetanide (▲), and negative controls as activated Rb^+ influx in the absence (Δ) and presence of 1 uM of digoxin (□).



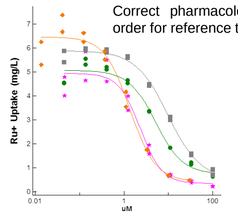
The window of detection determined by activated (2 min) and basal influx providing supportive evidence to its sensitivity. Error bars represent standard error of n=48.

Test Compound	AAS Based (Rb^+ Uptake) ICR8000™ IC_{50} (μM)	AAS Based (Rb^+ Uptake) ICR12000™ IC_{50} (μM)	^{86}Rb IC_{50}(μM)	Same Rank order for all 3 assays
4636277	0.72	0.89	0.3	1
Bumetanide	1.16	1.17	1.5	2
993437	1.76	1.6	5.9	3
4653400	4.18	5.16	12.0	4

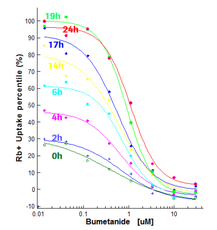
All 3 assays produced IC_{50} values in the same rank order. The rank order determined by ICR8000™ matched that of the high throughput system ICR12000™ which was also supported by the radioactive $^{86}Rb^+$ flux assay.

Assay optimization:
The assay was transferred to Roche, reference tool compounds test showed correct pharmacology and same rank order. The optimal doxycycline induction time is between 19-24 hr.

Correct pharmacology and same rank order for reference tool compounds

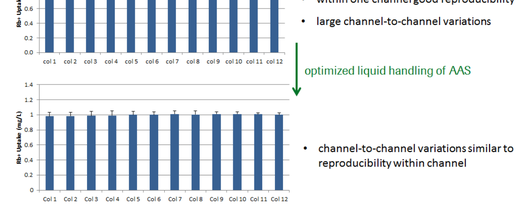


IC50 (uM)	Cerep Rb86 Assay (A7r5 cells)	Aurora	Roche
A	0.3	0.9	1.6
Bumetanide	1.5	1.2	1.3
B	5.9	1.6	3.4
C	12	5.2	7.5

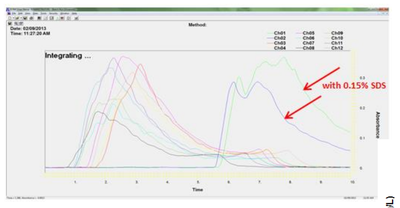


Doxycycline induction time course

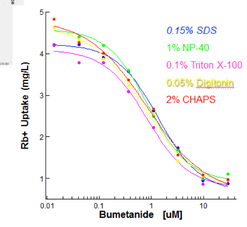
Optimized liquid handling by increasing sample volume improves readout:



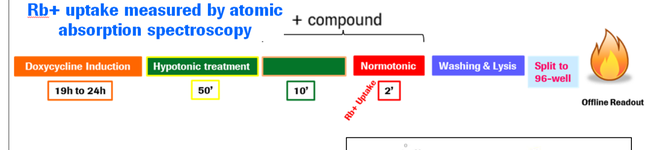
Cell lysis reagent optimization



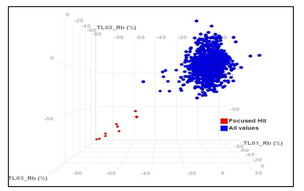
Replacement of SDS with NP40 for cell lysis solved the clogging issue inside the Aurora reader channel

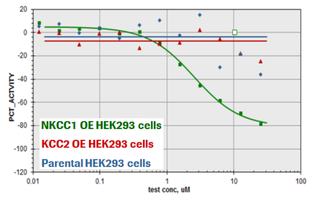


HTS screening campaign: Further automation optimization led to a complex protocol with hypotonic treatment, usage of two compounds copies, multiple washing, 384-well assay plate split into 4 96-well plates for offline reading:

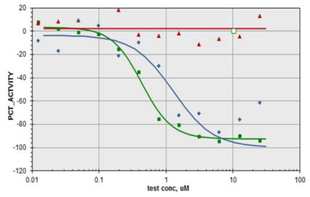


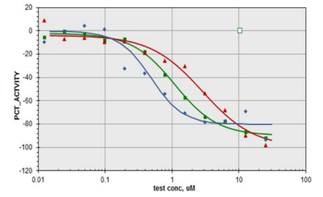
A focused library of 1450 compounds was screened in 3 independent runs for NKCC1 inhibitors. Robust results were achieved with a Z' factor over 0.7. Hit rate is 0.7% @ 40% cut-off



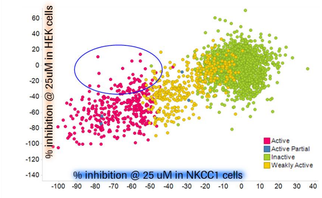


A full HTS campaign was carried out with 1.2 million compounds. Several chemical classes of specific hits were identified and confirmed with dose response confirmation. 6% of confirmed primary actives achieved selectivity.

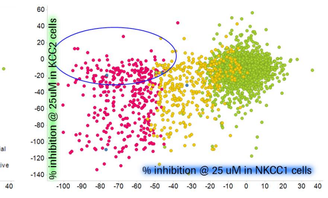




Hit confirmation



Hit selectivity



V. Conclusion

- The Rb^+ flux assay is a robust assay for high throughput screening of novel NKCC1 modulators
- Specific NKCC1 modulators were identified
- The Rb^+ flux assay is a good screening approach for non-electrogenic transporters

*Correspondence: yang.wen@roche.com, info@aurorabiomed.com

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