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DR ALVARO CORTES CABRERA (Orcid ID : 0000-0003-4635-6267)

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Identification of NEK3 and MOK as novel targets for lithium

Ana Bravo[1,2], *Héctor de Lucio*[2], *Pedro A. Sánchez-Murcia*[1], *Antonio Jiménez-Ruiz*[2], *Paula M. Petrone*[3], *Federico Gago*[1], *Alvaro Cortés Cabrera*[1,3*]

[1]Área de Farmacología, Departamento de Ciencias Biomédicas, Unidad Asociada al IQM-CSIC, Universidad de Alcalá, E-28805, Alcalá de Henares, Spain.

[2]Departamento de Biología de Sistemas, Universidad de Alcalá, E-28805 Alcalá de Henares, Spain.

[3]Pharma Research & Early Development (pRED). Roche Innovation Center Basel, Basel, Switzerland.

[*]alvarocortesc@gmail.com

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Running head: Lithium targets NEK3 and MOK.

ABSTRACT

Lithium ion, commonly used as the carbonate salt in the treatment of bipolar disorders, has been identified as an inhibitor of several kinases, including Glycogen Synthase Kinase-3β, for almost 20 years. However, both the exact mechanism of enzymatic inhibition and its apparent specificity for certain metalloenzymes are still a matter of debate. A data-driven hypothesis is presented that accounts for the specificity profile of kinase inhibition by lithium in terms of the presence of a unique protein environment in the magnesium binding site. This hypothesis has been validated by the discovery of two novel potential targets for lithium, namely NEK3 and MOK, which are related to neuronal function.

MANUSCRIPT

Protein kinases are key enzymes in many cellular signaling pathways and their dysregulation has been associated to a wide variety of pathological conditions in humans[1-3]. The kinase catalytic domain has a high degree of homology, with a largely conserved active site where the reversible transfer of a phosphoryl group from an adenosine triphosphate (ATP) molecule to the hydroxyl group of a specific serine, threonine or tyrosine residue is catalyzed. Such reaction depends on the presence of two magnesium ions that bind to two specific sites only available when the ATP-kinase complex is formed. Due to this dependency on magnesium, it has been found that other cations including Li^{+}[4] and Be^{2+}[5], can inhibit *in vitro* and in a Mg^{2+}-competitive manner the activity of kinases such as GSK3β, a serine-threonine kinase

involved in Wnt signalling[6] and neuronal function[7]. Although initially considered a highly selective GSK3β inhibitor, lithium ion has been described as an inhibitor of many other kinases, including MNK1 or HIPK3[8]. However, it has also been confirmed that an even larger group of kinases, such as cAMP-dependent protein kinase (PKA), casein kinase 2 (CKII) or mitogen-activated protein kinase 3 (MAPK3) are not affected by Li^+.

Lithium, usually administered orally as the carbonate salt, is used as a very efficient mood stabilizer for bipolar disorder treatment. It has been hypothesized that inhibition of GSK3β and inositol monophosphatase[9], together with other mechanisms such as alteration of glutamate receptors, could be responsible for the clinical effects of lithium ion[10, 11]. Although extensive work has been carried out in the area of lithium polypharmacology for years, its kinase selectivity profile remains an open question. Previous theoretical studies[12] on selectivity among the Mg^{2+}-containing metalloenzymes led to the conclusion that the net charge of the protein-magnesium complex and the solvent exposure of the magnesium binding sites were determining factors to explain why Li^+ versus Mg^{2+} competition was only possible in certain enzymes. However, as is often the case, "the devil is in the details", and such a general theoretical framework might be difficult to apply to the kinase selectivity problem, given that the active site in these enzymes is largely conserved.

To provide the necessary level of detail for this issue, we decided to focus our attention on the surroundings of the GSK3β-Mg^{2+} binding sites by building a model with the amino acid sequences of almost 80 different kinases that have been evaluated *in vitro* for lithium inhibition[8]. The Mg^{2+} binding site 1 (MGS1) or the right-hand site (Figure 1) involves the oxygen atoms of the ß and γ phosphoryl groups of the ATP molecule, the carboxylate of Asp200 (numbering scheme according to GSK3β), and three water molecules to complete the most common Mg^{2+} coordination number, which is 6[13]. The Mg^{2+} binding site 2 (MGS2), or the left-hand site, is made up by oxygen atoms from the α and γ phosphoryl groups, the

carboxylate of Asp200, the carboxamide oxygen of Asn186, and a variable number of water molecules depending on the stage of the reaction. Lu et al.[14] studied, from a theoretical standpoint, the possible pre-reactive complexes that Li^+ could produce by competing with the Mg^{2+} ions, and found significant changes in the geometry that could render the transfer of the phosphate impossible[14]. Additionally, it is also accepted that the stable form of the ATP molecule in biological fluids incorporates already at least one of the two Mg^{2+} ions required for the reaction, and that the binding energy of Mg^{2+} to ATP largely exceeds that of Li^+[15]. Taking together this information and in light of the recent structural findings by Taylor et al.[16] regarding the catalytic cycle of PKA, we argue that only the Mg^{2+} ion that binds at MGS1 can be recruited independently of the ATP molecule (Figure 1), and thus it is likely to be the only ion accessible to compete with Li^+.

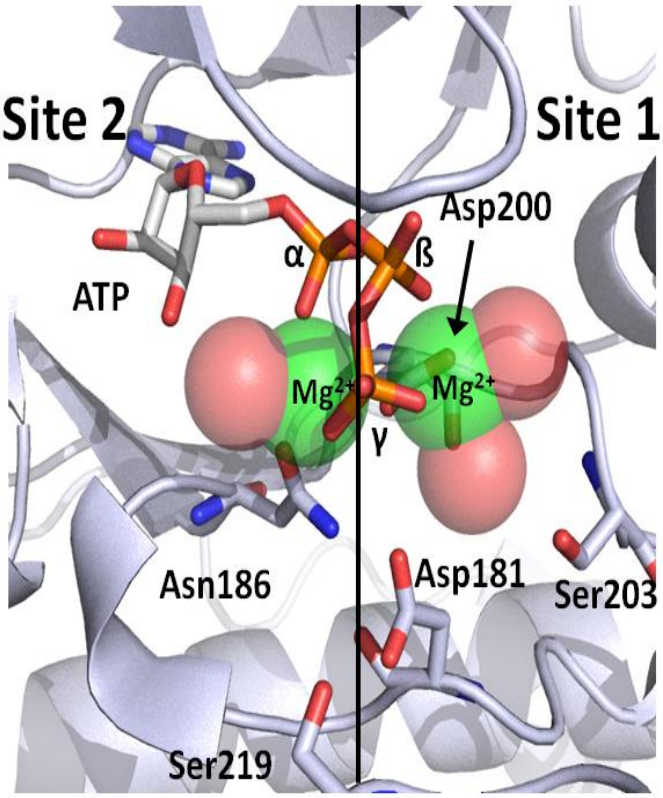


Figure 1. Mg^{2+}-binding sites. Mg^{2+} ions are depicted as green spheres, while the red spheres represent oxygen atoms from water molecules. Phosphate groups α, ß and γ are labeled with their respective Greek symbols.

We then proceeded to align the catalytic domains of all human kinases paying special attention to the surrounding of the MGS1 according to the available crystal structures of GSK3β[17] (Figure 1). Amino acids were reduced to 4 descriptors accounting for their size (volume), polarity (hydropathy index) and ability to form hydrogen bonds (donor or/and acceptor). A logistic regression was employed to build a model capable of identifying Li^{+}-sensitive kinases based on 78 sequences of MGS1 with known experimental results. The

model was then applied to the remaining kinases to identify new potential Li^+ targets. A total of 81 kinases were predicted, with a probability of more than 0.5, to be inhibited by lithium. From this set, we selected 5 kinases on the basis of both high confidence (probability ≥ 0.9) and availability for testing from our assay providers: CLK1, CLK2, MOK, NEK3 and TSSK4. We also selected another set of negatively predicted kinases for validation purposes (probability > 0.99 and assay available): CDK7, DMPK, MRCKβ, MSSK1, TAO2 and TAO3. Finally, and to provide internal controls for our assay, we tested three known Li^+-positive kinases, GSK3α, GSK3β and HIPK3, and three negative ones, PIM1, PIM2 and PKA.

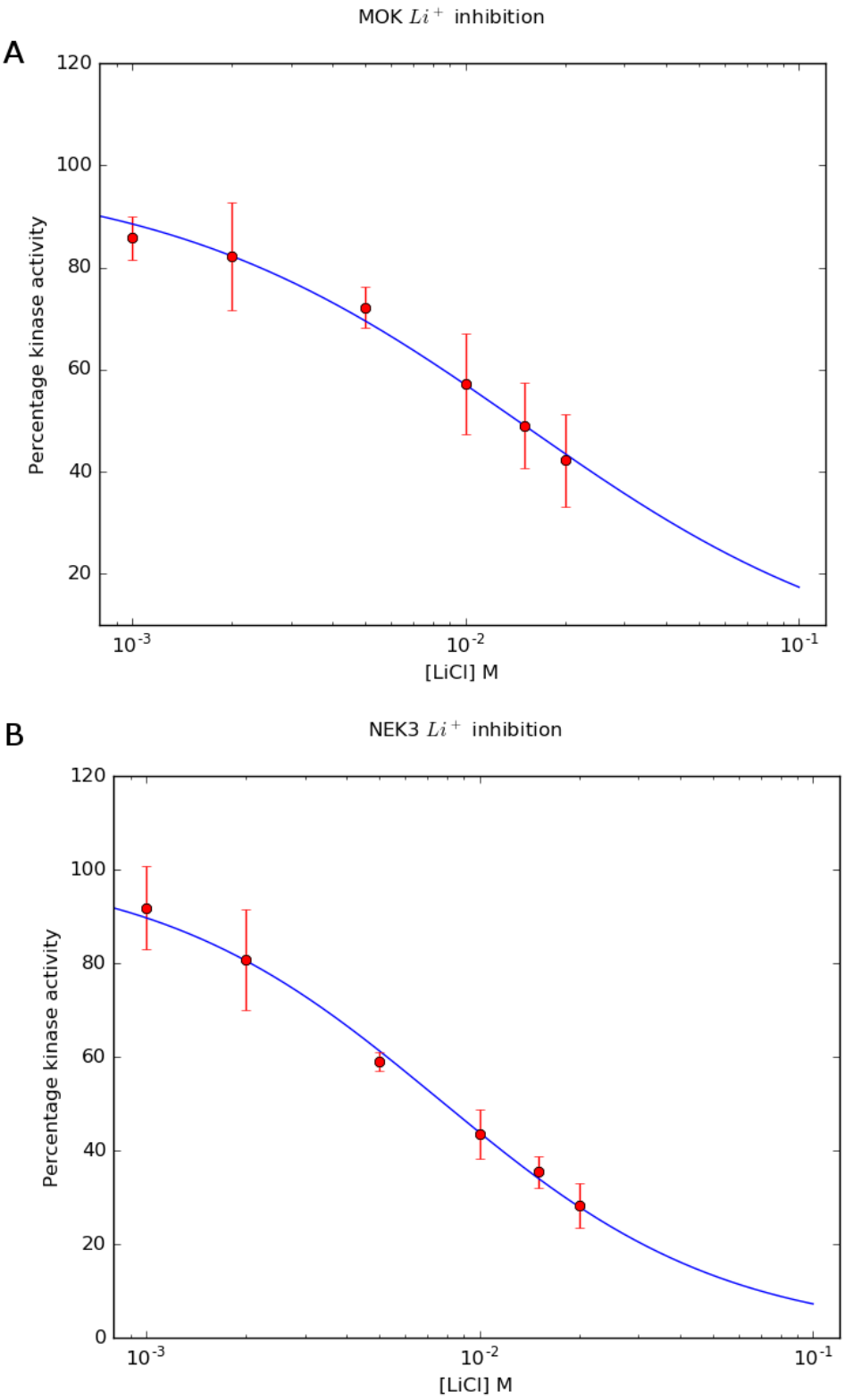
Inhibition curves were obtained using Li^+ concentrations ranging from 1 mM to 20 mM. Based on the profiles of PKA and GSK3β inhibition and the assay conditions, an enzyme was considered to be inhibited by lithium if, at a Li^+ concentration of 20 mM, the activity dropped below 70% that of the untreated control. Our results confirmed that GSK3α, GSK3β and HIPK3 were indeed affected by lithium (Table 1) and that PIM1, PIM2 and PKA were not. It is worth noting that the calculated IC_{50} value for GSK3β was approximately 10-fold higher than a previously reported value[9]. This fact is easily accounted for by the higher Mg^{2+} concentration that we have used in our assays and the known dependence of the inhibition on this cofactor[4, 18]. Remarkably, MOK and NEK3 were also found to be inhibited by lithium in a concentration-dependent manner and to a similar extent as was GSK3β (Table 1, Figure 2) whereas CDK7, DMPK, MRCKβ, MSSK1, TAO2 and TAO3, all predicted to be unaffected, did not show any significant inhibition at the Li^+ concentrations assayed. The fact that CLK1, CLK2 and TSSK4 were also found to be negative highlights the complexity of the underlying mechanism of kinase inhibition by lithium.

Regarding the newly identified lithium-sensitive kinases, NimA-related protein kinase 3 or NEK3, has been proved to influence neuronal morphogenesis and polarity through changes in microtubule acetylation, especially in the axons [19]. Remarkably, axonal disorganization in the prefrontal white matter has been identified as one of the early signs in patients with bipolar disorders [20]. MOK, also known as Renal antigen or RAGE, also appears to be more sensitive to lithium than is GSK3β. Interestingly, MOK has been found to phosphorylate the myelin basic protein (MBP) [21], which in turn has a major role in remyelination and brain development and function. Recent genenome-wide association studies have linked polymorphisms in the MBP gene with mood disorders and schizophrenia [22]. MOK also negatively regulates primary cilium growth [23] and the fact that lithium produces the opposite effect on neuronal cells[24] suggests that the observed lithium-induced phenotype could involve MOK inhibition. These NEK3- and MOK-mediated effects would support the idea of lithium as a regulator of the cytoskeleton of neurons, in agreement with several leading hypotheses in the bipolar disorder literature [25, 26]

Table 1. IC_{50} values for the kinases vulnerable to inhibition by lithium ion

Kinase	IC_{50}	Maximum inhibition (at 20mM)
GSK3α	14.5 ± 1.0 mM	59.8%
GSK3β	20.6 ± 1.6 mM	52.4%
HIPK3	>20 mM (24.0 ± 4.5 mM extrapolated)	47.6%
MOK	14.9 ± 1.4 mM	57.8%
NEK3	7.6 ± 0.5 mM	71.8%

Figure 2. Li^+ inhibition curves for A) MOK and B) NEK3.



Encouraged by the success in predicting Li^+ inhibition for MOK and NEK3 entirely on the basis of amino acid sequence of the putative MGS1, we decided to incorporate the results from our assays into our model and proceeded to deconvolute it by extracting the approximate importance of each amino acid position in the classifier (coefficients in the decision function). Interestingly, the single most important feature in the model corresponds to Tyr216, which is known to play a key role in GSK3β regulation[27]. Once phosphorylated, GSK3β becomes increasingly active via conformational rearrangements in the nearby zone. This is also the case for its counterparts Tyr161 in MOK[21] and Tyr359 in HIPK3[28], while Thr165 seems to regulate NEK3 in a similar manner[29]. The idea of a phosphate group located in close proximity to the Mg^{2+} binding site could suggest improved solvent accessibility in the active conformation of the kinase, which would make the MGS1 more prone to competition and displacement by Li^+. The list of important features is completed then (Figure 3) with Asn64, Phe67 and Gly68 in the G-loop; Phe201, located in the DFG motif; and His179, Arg180 and Ile182, which form the bottom of the MGS1. These residues, which make up a sort of cage, can be found in direct contact with the Mg^{2+} or Mn^{2+} ions in the MGS1 of some crystallographically solved kinases[30], or interacting with the first hydration layer of these cations so that they are expected to contribute heavily to the binding affinity for Mg^{2+} and Li^+ (see Supporting information for details).

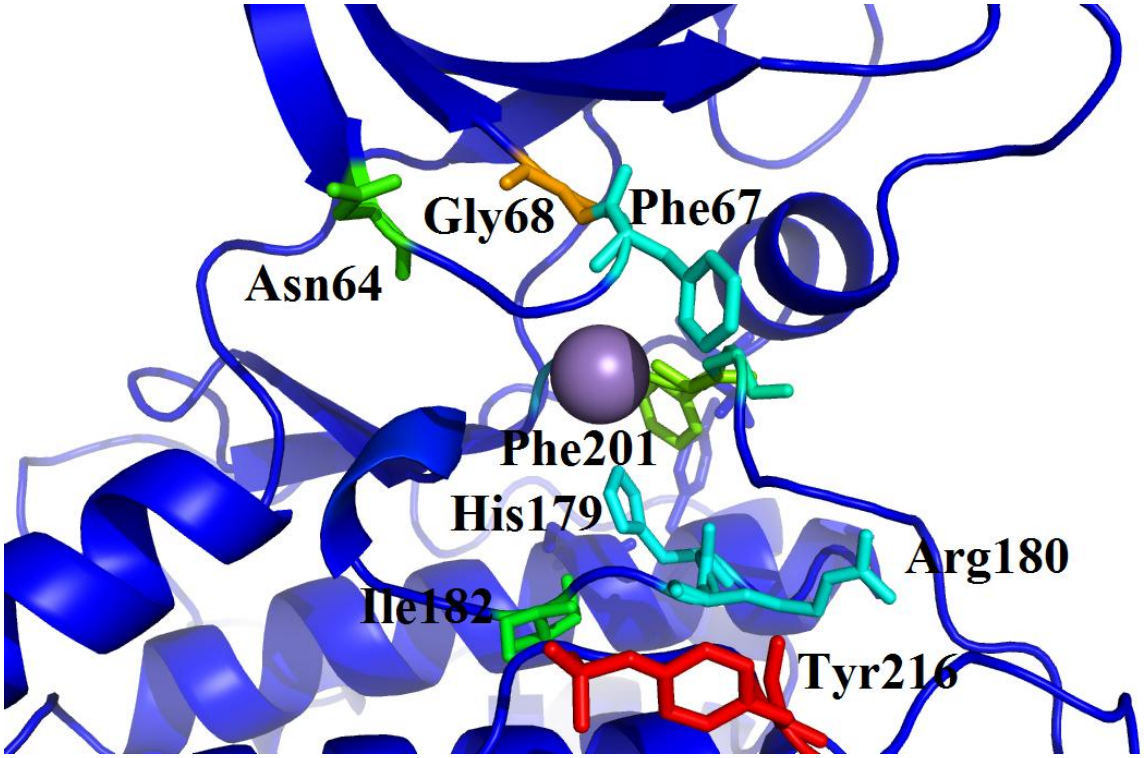


Figure 3. Color-coded structure of GSK3β (PDB 1J1B with a modelled P-Tyr216 residue) according to the importance of each residue in the classification model (red for most important residues to dark blue for residues not considered in the model). Tyr216 is the most important residue followed by residues surrounding the Mg^{2+} binding site 1: Phe67 (in the G-loop); Phe201 (in the DFG motif); and His179, Arg180 and Ile182 (bottom of the pocket).

In this work, we expand our current knowledge about the inhibition of kinase activity by lithium ions making use of machine learning algorithms. The proposed hypothesis is based on the configuration of the MGS1 in several kinases other than GSK3β, which in turn may be related to differences in solvent accessibility.

MATERIALS AND METHODS

Model and descriptors. A total of 413 protein sequences of kinase catalytic domains from the human kinome were downloaded from KinBase[31]. Kalign[32] was used for sequence alignment and GSK3β was used as the reference kinase. Any amino acid position that corresponded to a gap in the aligned GSK3β sequence was removed from the set prior to calculation of the descriptors. Only residues in the GSK3β 3D structure (PDB 1J1B) in a sphere of 12 Å around magnesium binding site 1 were considered. Volume (in $Å^3$)[33], hydropathy index (Kyte-Doolittle scale)[34] and the presence of hydrogen bond donors and acceptors (1 = present; 0 = not present) were used as descriptors for all amino acids in the alignment. Data from Bain et al.[8] was used as the training set to build a model capable of identifying kinases that can be inhibited by lithium, setting a threshold of a minimum of 40% inhibition at [LiCl] = 10 mM to consider a kinase as affected (see Supporting Table 1).

Kinase panel. Anhydrous lithium chloride >99% purity was acquired from Sigma-Aldrich. Kinase Profiler assays from Eurofins were used for the lithium screening based on radiometric [^{32}P]-ATP principles (see Supporting Table 2 for details). The lithium salt was dissolved in pure DMSO and used to screen in triplicate the different kinases at the following concentrations: 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, and 20 mM. Limited lithium solubility and assay protocols prevented the use of higher concentrations. Curves were fitted using a custom python script, the Hill equation and the module curve_fit from scipy.

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Conflict of Interest: ACC and PMP are former F. Hoffmann-La Roche employees.

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