BS8 RAF 'PARADOX BREAKER' PLX8394 ACTIVATES ERK1/2 VIA CRAF, WITH NO EFFECT ON ANGII-INDUCED CARDIAC REMODELLING IN MICE

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Introduction The ERK1/2 cascade, a key pathway involved in cardiac remodelling, is regulated by RAF kinases. Small molecule inhibitors of RAF have been developed due to activating oncogenic mutations, however paradoxical activity has been seen in early generations of inhibitors. Therefore, 'paradox breaker' inhibitors (e.g. PLX8394) have been developed and are undergoing clinical trials. Here, we investigated the effects of PLX8394 on vascular ERK1/2 signalling in vitro and on hypertensive cardiac remodelling in vivo.

Methods Murine endothelial cells (ECs) or human cardiac fibroblasts (HCFs) were incubated with PLX8394 and effects on RAF-ERK1/2 pathway activity determined by western blotting, with effects on cell migration and proliferation assessed via wound healing and BrdU assays. For in vivo characterisation, PLX8394 (5mg/kg/d) was infused with/without angiotensin-II (AngII; 0.8mg/kg/d) for 7 days by osmotic minipumps in male wildtype C57Bl/6J mice (n=8–11/group). Cardiac function/dimensions were assessed using echocardiography; effects on cardiac morphology were assessed by histological staining. mRNA expression was assessed by qPCR. Statistical tests used 1-way ANOVA with Holm-Sidak's post-test.

Results PLX8394 (5 min; 1uM) activated ERK1/2 (n=3; p=0.018) pathway via CRAF (n=3; p=0.047) in ECs with no change seen in BRAF activity. This was accompanied by increased BrdU incorporation (n=6; p=0.0002; p=0.0009) but significantly inhibited migration (n=6; p<0.0001; p<0.0001) both at baseline and with AngII (100nM), respectively. In HCFs however, PLX8394 had no effect on baseline or AngII migration (n=4; p=0.99; p=0.98) or BrdU incorporation (n=6; p=0.95; p=0.65). In vivo, PLX8394 did not alter the AngII-induced cardiac hypertrophy with maintained wall thickness to internal diameter ratio (p=0.45). While PLX8394 was able to significantly reduce cardiomyocyte cross sectional area (p=0.0068), no changes were seen in Myh7, Nppa or Nppb mRNAs. Moreover, PLX8394 did not significantly alter the perivascular (p=0.69) or interstitial (p=0.052) fibrotic area with no changes in mRNA expression of collagens1–4.

Conclusion PLX8394, despite development as a cancer cell 'paradox breaker', activates ERK1/2 signalling in ECs, but not HCFs. In vivo, PLX8394 had minimal effect on hypertensive cardiac fibrotic remodelling despite reducing myocyte hypertrophy, likely reflecting a cell-type dependent response. Thus, paradox-breaker RAF inhibitors, currently in clinical trials for RAF-mutant cancers, may have limited viability as hypertension therapies.

BS9 LONG QT SYNDROME-ASSOCIATED CALMODULIN MUTATIONS AFFECT INTERACTION WITH L-TYPE CA2+ CHANNEL (CAV1.2) AND CAMKIIΔ ACTIVITY

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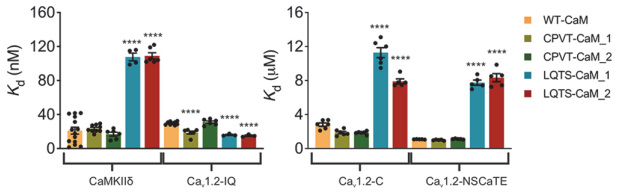
Introduction Catecholaminergic polymorphic ventricular tachycardia (CPVT) and Long QT Syndrome (LQTS) are two major inherited lethal cardiac channelopathies responsible for sudden death predominantly among the young population. Of late, mutations in the highly conserved calcium (Ca2+) sensor protein calmodulin (CaM) have been linked to these cardiac arrhythmias in human patients. CaM regulates the activity of ion channels such as the Voltage-Gated Calcium Channel (Cav1.2) involved in cardiac muscle contraction either via direct binding or indirectly through Ca2+/CaM-dependent protein kinase II (CaMKII□) activation. However there is a major gap in the understanding of the precise molecular mechanism by which CaM mutations contribute to irregular heartbeats.

Methods Short synthetic peptides encompassing the CaM binding regions were used for the characterisation of CaM-target interaction (CaMKII□294–315, Cav1.2-NSCaTE51–68, Cav1.2-IQ1665–1685 and Cav1.2-C1627–1652). The binding parameters of CaM with each of these peptides in Ca2+ saturated conditions (5 mM CaCl2) were measured using isothermal titration calorimetry (ITC). The atomistic details of binding were obtained through X-ray co-crystallization experiments. CaMKIIδ phosphorylation activity was measured using western blot and fluorescence kinase assay.

Results Binding affinity of CaMKIIδ and Cav1.2 peptides to the CPVT-associated CaM variants were comparable to WT-CaM. However, the affinity of LQTS-associated CaM variants was reduced up to 5-, 7- and5-fold for CaMKIIδ294–315, Cav1.2-NSCaTE51–68 and Cav1.2-C1627–1652 respectively. Interestingly, the Cav1.2-IQ1665–1685 peptide showed a stronger binding, up to 2-fold, towards LQTS-CaM mutants. Crystal structures of CaM in complex with CaMKIIδ showed structural alterations induced by LQTS associated mutations. In addition, we demonstrated that LQTS-associated CaM mutants can decrease CaMKIIδ autophosphorylation and kinase activity.

Conclusions These data suggest a strong correlation between LQTS-associated CaM mutations and Cav1.2 dysregulation, either through impaired interaction or altered CaMKIIδ activation. However, CPVT-associated mutations do not seem to affect the interaction with either Cav1.2 or CaMKIIδ, suggesting that the disease manifestation might involve other CaM targets. We conclude that disease-associated mutations have unique molecular mechanism of action which defines the type of resulting calmodulinopathy.



Affinity of the binding of CPVT-associated CaM mutants (CPVT-CaM_1, CPVT-CaM_2) and LQTS-associated CaM mutants (LQTS-CaM_1, LQTS-CaM_2) to CaMKIIδ$_{294-315}$, Ca$_v$1.2-IQ$_{1665-1685}$, Ca$_v$1.2-C$_{1627-1652}$ and Ca$_v$1.2-NSCaTE$_{51-68}$ peptides. Data were obtained using ITC and fitting to a one-site binding model. Data were processed using the MicroCal PEAQ-ITC software. Data are mean±s.e.m. Experiments were performed in the presence of 5 mM CaCl2 at 25°C. ****P<0.0001 (versus CaM-WT; differences between groups were determined using one-way ANOVA with Dunnett's post-hoc test).

Abstract BS9 Figure 1