An integrated anti-arrhythmic target network of compound Chinese medicine Wenxin Keli revealed by a combined machine learning and molecular pathway analysis

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7mb00003k

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Abstract: Wenxin Keli (WK), a Chinese patent medicine, is known to be effective for cardiac arrhythmias and heart failure. Although a number of electrophysiological findings regarding its therapeutic effects have been reported, the protective effect of WK on suppressing anti-arrhythmic agents induced arrhythmias has not, neither

have the system-level characterizations of the component-target interactions of WK been elucidated. In the present study, we recorded the pseudo-ECGs of isolated guinea pig hearts, both with and without WK, when quinidine was applied to change the heart rate and prolong the Q-T interval. The hearts that were given WK were far less effected by the quinidine. To explain the therapeutic and protective effect of WK, we constructed an integrated multi-target pharmacological mechanism prediction workflow in combination with machine learning and molecular pathway analysis. This workflow had the ability to predict and rank the probability of each compound interacting with 1715 target proteins simultaneously. The ROC value statistics show that 97.786% of the values for target prediction were larger than 0.8. We applied this model to carry out target prediction and network analysis for the identified components of 5 herbs in WK. 124 potential anti-arrhythmic components with 30 corresponding protein targets were obtained, and an integrative anti-arrhythmic molecular mechanism of WK was proposed. Calaxin, one of the 124 components, was used to test the Cav1.2 channel with an electrophysiological assay. The result showed that Calaxin can block the Cav1.2 channel dose-dependently, which confirmed the predicted results and partially explained the action mechanism of WK.

Keywords: Wenxin Keli, anti-arrhythmia, ECG, machine learning, network pharmacology, IPA

Introduction

Arrhythmia is a disease featuring the abnormalities of frequency or rhythm of heart excitement. Based on the heart rhythm, arrhythmia can be classified into tachyarrhythmia (e.g. atrial fibrillation) and bradycardia (e.g. sick sinus syndrome). A variety of anti-arrhythmic drugs have been discovered and developed and the most commonly used are chemically synthetic in nature. However, modern Chinese herbal medicines, which are developed according to traditional Chinese medical theory, also play an irreplaceable role in the treatment of cardiac arrhythmias.

WK is one of the most widely prescribed antiarrhythmic patent Chinese medicine in China. It is composed of extracts from 5 herbs: *Nardostachys jatamansi* DC (Gansong), *Radix Notoginseng* (Sanqi), *Succinum* (Hupo), *Polygonatum sibiricum* (Huangjing) and *Codonopsis pilosula (Dangshen).* A randomized, double-blind, placebo-controlled, parallel-group, multicenter clinical trial of Wenxin Keli with 1200 eligible participants for 4-weeks was reported in 2015.[1] It showed that WK effectively reduced the overall number of patients with premature ventricular contractions (PVCs) and alleviated PVC-related symptoms. The total effective rate was 83.8% and no severe side effects were detected. Another clinical trial reported by Meng and co-workers demonstrated that WK assisted sinus rhythm (SR) reversion from hyperthyroidism-caused paroxysmal atrial fibrillation and maintained SR in such patients.[2] A recently published Expert Consensus on WK for treatment of cardiac arrhythmias has defined its clinical application[3]. Despite of its wide use for the treatment of cardiac arrhythmia in China, our knowledge of the pharmacological mechanisms of WK remains limited.[4-6] In 2012, Antzelevitch's group reported for the first time that WK significantly reduced Nav1.5 channel in HEK293 cells stably expressing SCN5A and revealed later in a follow-up study that WK binds to the inactivated state and dissociates from the closed state of Nav1.5 channel very rapidly.[5, 7] In addition, late Sodium channel, L-type Calcium channel, L-type calcium channel, transient outward potassium channel and calcium/calmodulin-dependent protein kinase II signal transduction pathway were also reported under the regulation by WK.[8-11] Unlike a typical single-molecule chemical drug, the chemical composition of a Chinese medicine formula is complex and likely contains multiple components capable of interacting with multiple targets.[12] As the process of TCM pharmacological research is rather complicated and the research approaches are insufficient, therefore, the global pharmacological (especially non-electrophysiological) mechanism of WK has not been well discovered.[13-17]

TCM compound formula are well known by their adoption of multiple components to take multi-pharmacological effects on multiple targets.[18] However, the complexity of the chemical composition of compound TCM formula brings great difficulties to the pharmacological research. In recent years, network pharmacology

has become one of the most widely used approaches to reveal pharmacological mechanism of compound TCM formula.[19] Network pharmacology is an effective approach to address the relationship between multiple components and drug synergistic effects on a series of targets in the pharmacological network.[20] Numerous of classic and modern formulae have been studied by network pharmacological approach, such as Chaihu-Shu-Gan-San,[21] Fufang Xueshuantong Capsule,[22] ShengMai preparations,[23] Xiao-Ke-An,[24] Modified-Simiaowan,[25] Xue-Zhi-Ning,[26] QiShenYiQi formula,[27] Bushenhuoxue formula,[28] Gansui Banxia Tang,[29] Ge-Gen-Gin-Lian decoction,[30] Liu-Wei-Di-Huang pill,[31] Ma-huang Decoction,[32] Si-Wu-Tang,[33] Tianshu formula,[34] Qing-Luo-Yin,[35] Xiao-Chaihu-Decoction[36] and Zhi-Zi-Da-Huang decoction,[37] et al. The key procedure of network pharmacological research is the establishment of the relationship between chemical compounds and their targets (Drug-Target Relationship). Based on the type of core algorithm on generating drug-target relationship, network pharmacological research on Chinese medicine can be divided into three categories: virtual screening (e.g. Molecular Docking,[22, 28, 37-39] pharmacophore,[23, 40]) literature mining,[25, 41] and machine learning.[42-44] Literature mining is the most accurate in all of them, however the disadvantage of which is low efficient and could not be overcome. The operational efficiency of the virtual screening approaches and machine learning is in the same magnitude, but the accuracy of the latter is much higher than the former. However, in most of the research reports with machine learning approach, only a small number of targets were studied without investigating the effects of the drug in the full range of human targets.

In this paper, we selected Naïve Baysian Model (NBM) as the machine learning core algorithm, as a classic binary classification algorithm Naïve Baysian Model is capable of each chemical component interacts or not interacts with each target. Compared to other algorithms, such as artificial neural networks (ANN), support vector machine (SVM) etc., the Bayesian model has three important features: first, it is high efficient for large datasets, scaling linearly with respect to the number of molecules; second, NBM is less affected by multiple dimensions even large numbers of descriptors are used; third, the Bayesian model weights features by assigning

greater significance to characteristics that appear to distinguish good samples from baseline samples.[45] To investigate the integrated anti-arrhythmic mechanism of WK, firstly we tested the protective effect of WK on suppressing the arrhythmogenic effect of quinidine, and then we established a database for the chemical components of the 5 herbs in WK, predicted the component-target spectrum for each component with the full range of human targets by machine learning, and overlap the component target spectrum with the disease target spectrum of cardiac arrhythmia generated by IPA. In accordance with the overlapping portions of the two target spectrums, we marked out the components with higher "active" possibility of the 5 herbs in WK and analysed the final target spectrum of overlapping network for the active components. Finally, we proposed an integrated pharmacological mechanism to elucidate the protective and therapeutic effect of WK and tested one of the component Calaxin from Gansong by electrophysiological assay.[46]

Materials and methods

Animals

Hartley guinea pigs (350～400 g) were purchased from the Academy of Military Science of the Chinese People's Liberation Army (SCXK-(Jun) 2012-0004). The rodents were housed in cages at a temperature of 22°C±2°C and humidity 40%±5%, under a 12-hour light/dark cycle, and received standard diet and water ad libitum. All experiments were reviewed and approved by the Committee of Ethics on Animal Experiments at the TJAB (TJAB-JY-2011-002) and were carried out under the Guidelines for Animal Experiments at the Tianjin University of Traditional Chinese Medicine.

Drug and reagents

WK was obtained from Shanxi Buchang Pharmaceutical Co., Ltd. (Shanxi, China) and quinidine was purchased from J&K Scientific Ltd. (Beijin, China), both were dissolved in saline. CHO cells were brought from ICE BIOSCIENCE INC. (Beijin, China). DMSO was purchased from Solarbio (Beijing, China) and other Reagent including NaCl, KH2PO4, MgSO4 are purchased from Sigma Chemicals (St. Louis, MO USA).

Isolated heart perfusion and electrical signal recordings

Fifteen Hartley guinea pigs (350~400 g, both sexes) were divided into 3 groups: WK-treatment group, Quinidine-treatment group, and WK+Quinidine-treatment group. The guinea pigs were anesthetized for 5~10 min, the hearts were excised and immediately immersed in cold Krebs-Henseleit (KH) solution contained the following (120 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose, 2.0 mM Na-pyruvate and 1.8 mM $CaCl_2$) The aorta was immediately cannulated to the Langendorff perfusion apparatus (Powerlab/8sp, ADInstruments Pty Ltd., Australia) and perfused retrogradely with KH solution (36.5 ± 1 °C, pH 7.35–7.40) at a constant flow mode. KH solution was equilibrated with 95% O_2 and 5% CO_2 mixed gas and the perfusion speed was adjusted to 10-15 mL/min to maintain the pressure between 70-80 mmHg. Experiment was initiated with a 15-min vehicle control period on all acceptable heart immediately after the stabilization period. All hearts were exposed to the vehicle control at the same DMSO concentration. After the perfusion of vehicle control, a compound testing solution was perfused immediately. The heart beat spontaneously at sinus rhythm. WK and quinidine were freshly dissolved in KH solution before drug application. Firstly, the equilibrated hearts were perfused with KH solution for 10 min to stabilize the heart beat and state. In control group, KH solution was continuously perfused for 10 min; in WK group, the hearts were perfused with 50mg/L WK for 10 min; in Quinidine group, perfused with 3.25mg/L quinidine for 10 min to induce long Q-T symptom; in W+Q group, 50mg/L WK was first perfused and 10 min later 3.25mg/L quinidine was added.

ECG (measured as a bipolar trans-ventricular electrocardiogram) was recorded through two surface electrodes held lightly with a spring against the epicardium. The positive and negative electrodes were relatively placed on the left ventricle in a position where a stable T wave can be obtained with a clear endpoint and on the right ventricle near the atrium-ventricle ring. All the data were collected by LabChart 7.0 (ADInstruments Pty Ltd., Australia), P-R interval, QRS interval, Q-T interval and heart rate were measured and analyzed.

General procedure for target prediction of WK

With the purpose of illustrating the complex pharmacological mechanism of WK, we established a methodology to predict the targets for complex component composition of WK and to pinpoint the components with antiarrhythmic activity and to establish the molecular pharmacology network of anti-arrhythmic effect of WK. The main process flow is shown in Fig. 1.

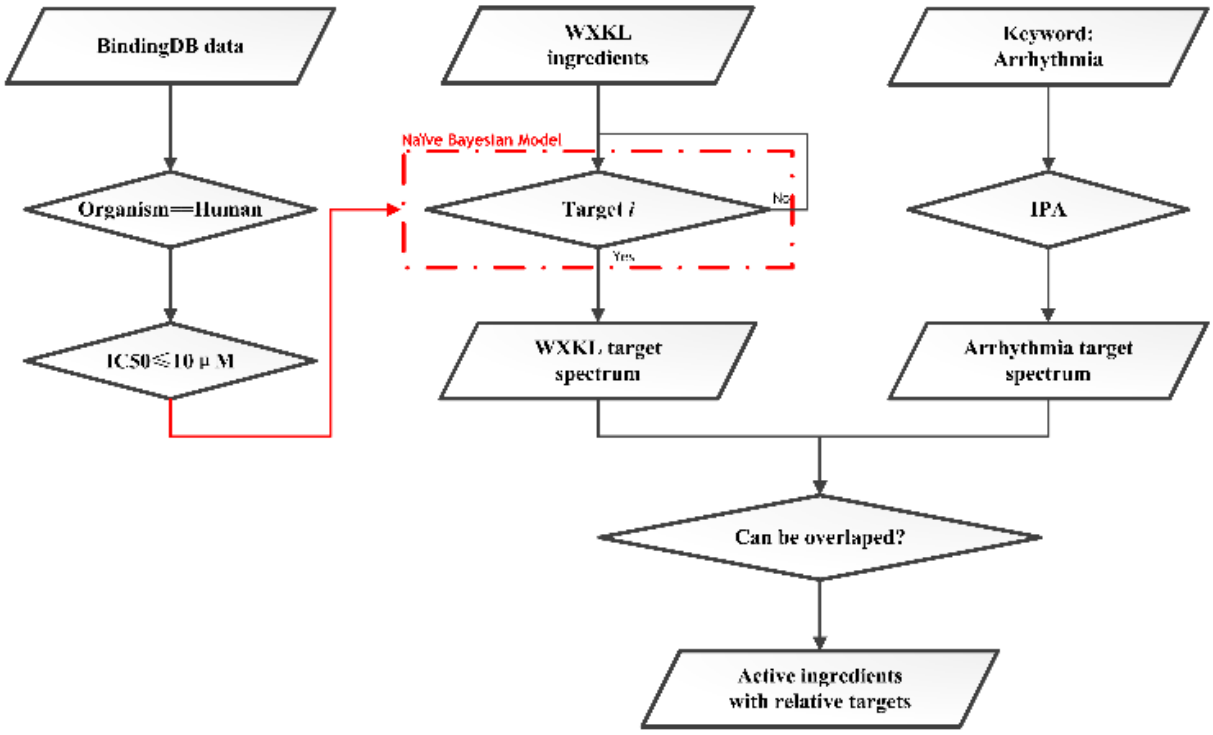


Fig. 1 Flowchart for target prediction model establishment and working procedure.

Data collection of 3D molecular structures of training set for establishing target prediction model and WK components

Firstly, for establishing target prediction model, we downloaded all of the experimental data from BindingDB database (http://www.bindingdb.org), isolated the structural information and activity data of compounds interacting with all human targets and filtered the EC50, IC50, Kd and Ki by a threshold of 10 μM, 599380 molecules left and were collected as a training set for model establishment.47-50 We collected all the components of Gansong, Sanqi, Hupo, Huangjing and Dangshen

from databases: TCMSP,[51] TCM@Taiwan,[52] Reaxys, TCMID,[53] HIT,[54] etc., and downloaded chemical structure of these components from PubChem database (http://pubchem.ncbi.nlm.nih.gov/), Chemspider database (http://www.chemspider.com/) and chemicalbook database (http://www.chemicalbook.com/) to establish the virtual compound database (WK component library) contained 475 components and 298 of 475 have three-dimensional structures.[52, 54, 55] The new compounds isolated by our laboratory have also been added into the library, part of the data in WK component library shown in Table S1, ESI†.[56]

Protocols establishment with Pipeline Pilot

Pipeline Pilot software (SciTegic, Inc., San Diego, CA) were used to establish virtual component library and component target predicting protocol based on Naïve Bayesian Classification model, and another protocol was used for overlapping the component and disease target networks to pinpoint the anti-arrhythmic components.

Each input record has a set of descriptors including a structural key fingerprint (FCFP_6) and physical properties such as molecular weight, number of rotatable bonds, and AlogP.[57-59] Based on these descriptors, a series of two-class Bayesian categorization model was established to predict each molecule in the testing set the possibility of interacting with some target protein. The algorithm orderly examines each descriptor to find the best classification parameters that can best separate the active compounds from the inactive ones. For binary fingerprints, the separation is defined by the presence or absence of some feature bit in the fingerprint; for continuous variables, the method examines numbers of bin boundaries as possible splits for each variable.

For each target prediction, a ROC curve was generated, and the area under the curve (XV ROC AUC) of false positive rate vs false positive rate was calculated to evaluate the performance of the binary Bayesian classifier model. [60, 61]

Ingenuity pathway analysis for building-up the arrhythmic target spectrum and WK pharmacological network

We searched the keyword “arrhythmia” through the use of QIAGEN’s Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), collected all the targets related to the disease to establish “arrhythmic target spectrum” with the network building function of IPA.[62, 63] Further on, the protocol mentioned before for overlapping the component target spectrum and arrhythmic target spectrum was used to mark out the common part, in which the targets were relatively correlated with the anti-arrhythmic components and was used for establishing pharmacological mechanism network of WK for the treatment of arrhythmia.

The electrophysiological recordings and data analysis

For recording Cav1.2 current, the extracellular Ringer’s solution contained: 140 mM TEA-Cl, 2 mM MgCl2, 10 mM CaCl2, 10 mM HEPES, 5 mM Glucose, pH=7.4; The pipette solution contained: 120 mM CsCl, 1 mM MgCl2, 10 mM HEPES, 4 mM Mg-ATP, 10mM EGTA, 0.3mM Na2-GTP, pH=7.2. An EPC10 amplifier was used to record the electrophysiological signal and data were stored and analyzed with Patchmaster and Igor Pro software. Cav1.2 currents were elicited by a 10 mV, 300 ms depolarized pulse from a holding potential of -60 mV. This voltage-clamp pulse protocol was performed continuously during the experiment. An inter-pulse interval of 20 seconds was used to allow the recovery from inactivation.

CHO cells were stably transfected with 3 plasmids with the subunit genes of Cav1.2 channel: CACNA1C (NM_000719), CACNB2 (NM_201597) and CACNA2D1 (NM_000722). Cav1.2 Cell was incubated with test compounds for 5 minutes, or until the current reached a steady-state level, then the Cav1.2 current was recorded. The test and control solutions were superfused into a recording chamber mounted on the stage of an inverted microscope via a gravity-fed solution delivery system. During the experiment, the outflow solutions were discarded by vacuum aspiration. All tests were performed at room temperature (25℃). The IC50 values for each compound were determined by dose–response curves with 5 concentration points.

Results

The protective effect of WK on suppressing the arrhythmogenic effect of quinidine

Compared with the control, WK does not significantly change the ECG shape, Quinidine not only prolonged the heart beat period, but also decreased the height of R wave, and however, with WK perfused before quinidine, the ECG shape was maintained as control group (Fig. 2A). As an arrhythmogenic agent, quinidine at concentration of 3.25mg/L significantly changed heart rate, QRS and QT interval. WK had no effect on heart rate, PR, QRS and QT interval but significantly prevent changes induced by quinidine. The combinative effect of W+Q group did not produce significant change on HR and QT interval when compared with the control group (Fig. 2).

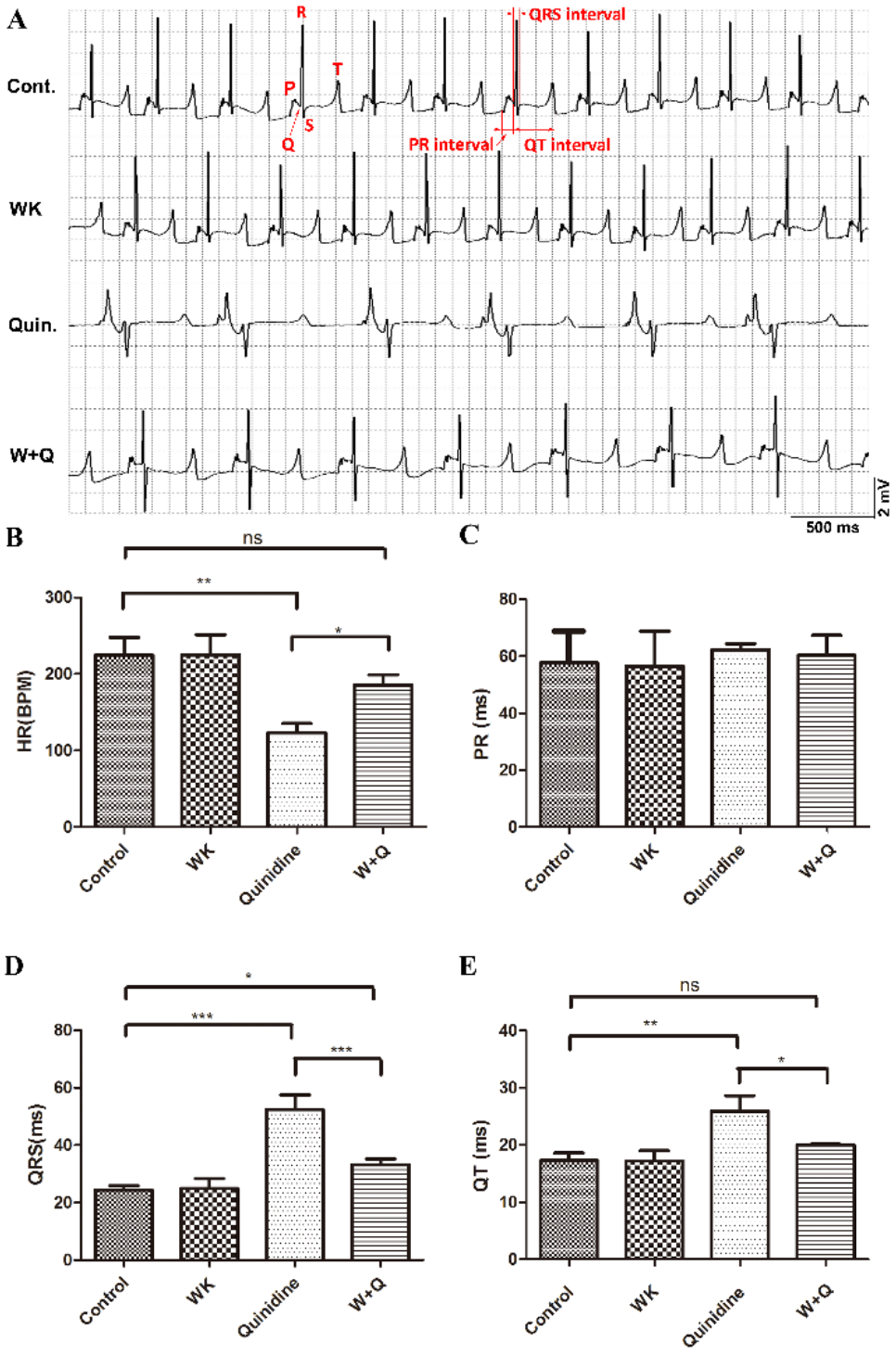


Fig. 2 Prevention of quinidine-induced arrhythmia by WK in isolated hearts from guinea pigs. **A)** Representative ECG recordings of vehicle control, WK, Quinidine and WK+quinidine treatment groups; Statistical analysis for **B)** Heart Rate (HR), **C)** PR-interval, **D)** QRS duration and **E)** QT interval of each groups**.** ns=not significantly different; * P<0.05, ** P<0.01, *** P<0.001, n=5.

Establishment of a multi-target prediction model

To achieve the above purpose, we designed a Pipeline protocol to recognize and isolate better activity data of human targets from BindingDB database (Fig. S1, ESI†). The activity data with IC50 greater than or equal to 10 μM and the non-human data were excluded, the remaining data set were prepared as positive molecules for establishing the Multi-target prediction model. We applied Naïve Bayesian Classification algorithm to build the protocol for establishing the target prediction model based on machine learning. This model was built using 599380 samples which were uploaded before December 2014, and was validated using a leave-one-out cross-validation. Each sample was left out one at a time, and a model built using the results of the samples; this model was then used to predict the left-out sample. The statistics for area under ROC curve (AUC) was shown as Fig. 3, 97.786% AUC values for target prediction were larger than 0.8.

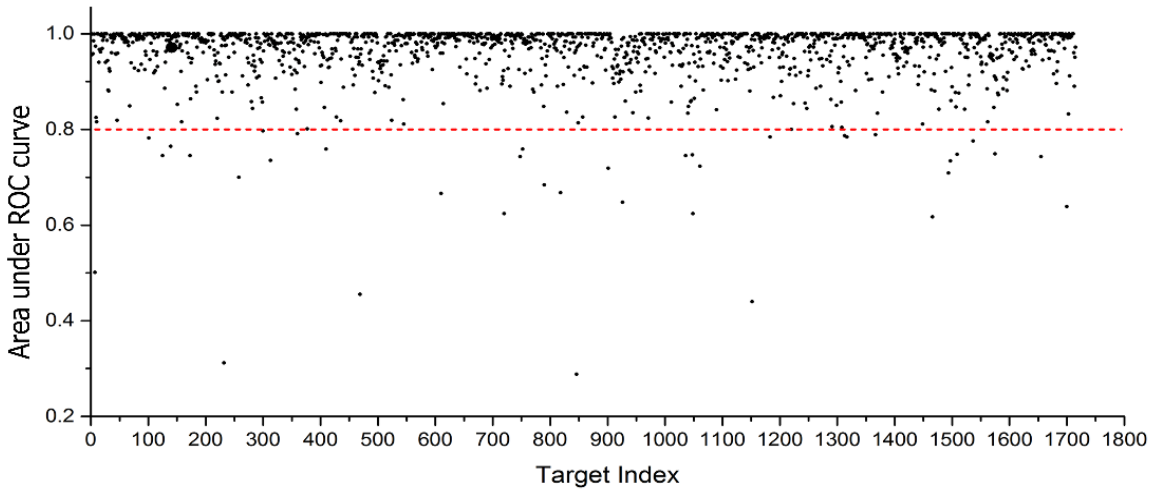


Fig. 3 Statistics for the Area under ROC curve of all targets. All 1715 targets were sorted alphabetically, and the area under the curve were calculated. The larger the area under the ROC curve, the higher the precision of prediction of the relevant target, 97.786% AUC values for target prediction were larger than 0.8.

Acquisition of component target spectrum of herbal compounds in WK

Another protocol was built to generate component target spectrum of the five herbs in WK using the multi-target prediction model mentioned before (Fig. S2, ESI†).

Except the 599380 molecules，all the data refresh before December 2015 were downloaded for evaluating the accuracy of the entire multi-target prediction model. 3 testing sets were established with 3000 molecules selected randomly. The true positive rate, false positive rate and accuracy value were calculated for each target score threshold to ensure to obtain the maximum degree of correct prediction (Fig. 4). The accuracy increases while Target_score increases with TPR and FPR decreasing. The highest accuracy appeared ACC=0.9026 when Target_score=10.0, which means more than 90% of the prediction results would be correct in the prediction model above. Finally, the threshold for outputting component-target relationship was set as 6.0, at which the accuracy was 0.8197, with a 65.27% true positive rate and 17.97% false positive rate. Based on the protocol above, the target spectrum of 298 components with 3D structures was predicted.

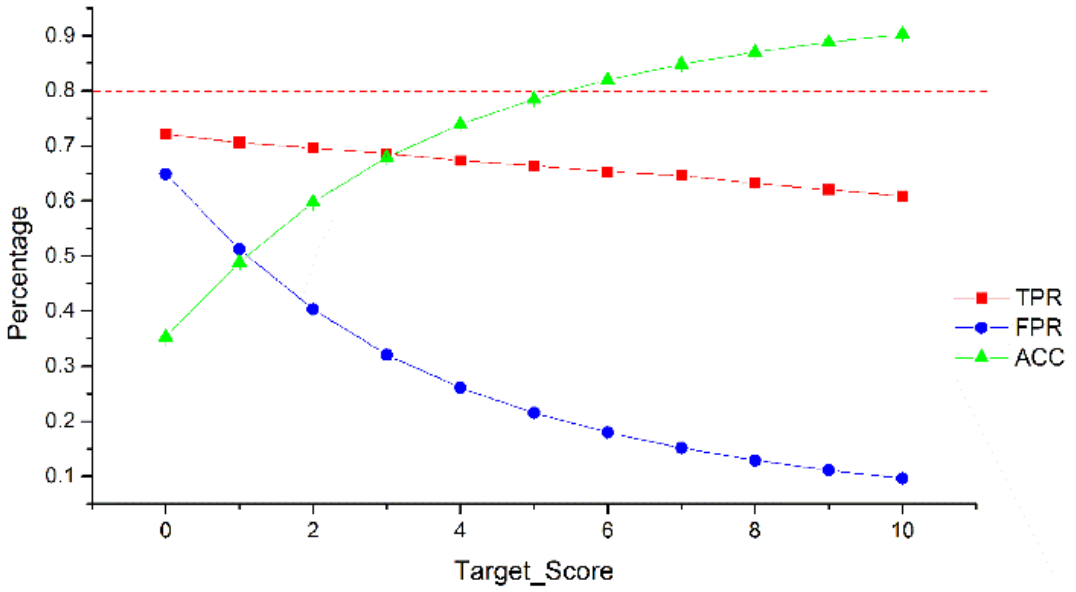


Fig. 4 The true positive rate, false positive rate and accuracy value for each target score threshold of the multi-target prediction model. With the increase in Target_score, the accuracy of the prediction model increased and both true positive rate, false positive rate decreased. However, the threshold of Target_score should not be compromised to keep enough samples in the prediction results, 6.0 was selected as the threshold, at which the accuracy was 0.8197. TPR: true positive rate, FPR: false positive rate, ACC: accuracy.

Disease target spectrum and active components

To narrow the target search range, we established a disease target set for filtering out the relationships which were not related to cardiac arrhythmia. Arrhythmic target information was obtained from IPA database with a keyword "arrhythmia", 153 targets related to arrhythmia were collected as arrhythmic disease target spectrum.

In order to display the arrhythmia target spectrum better, we established a disease target network map shown as figure 5. We used the comparison function of Pipeline Pilot software once more to overlap the disease target spectrum established with IPA and component target spectrum predicted above, the common part of the two spectra was kept and the components relevant to each target in the common part was obtained as the active components.

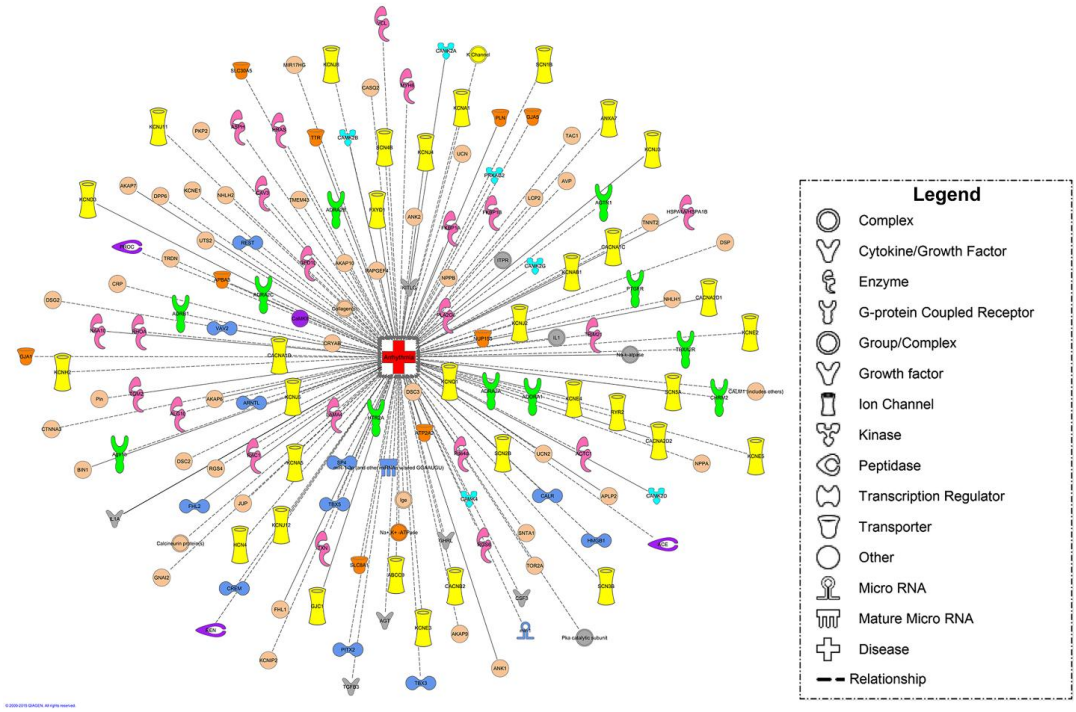


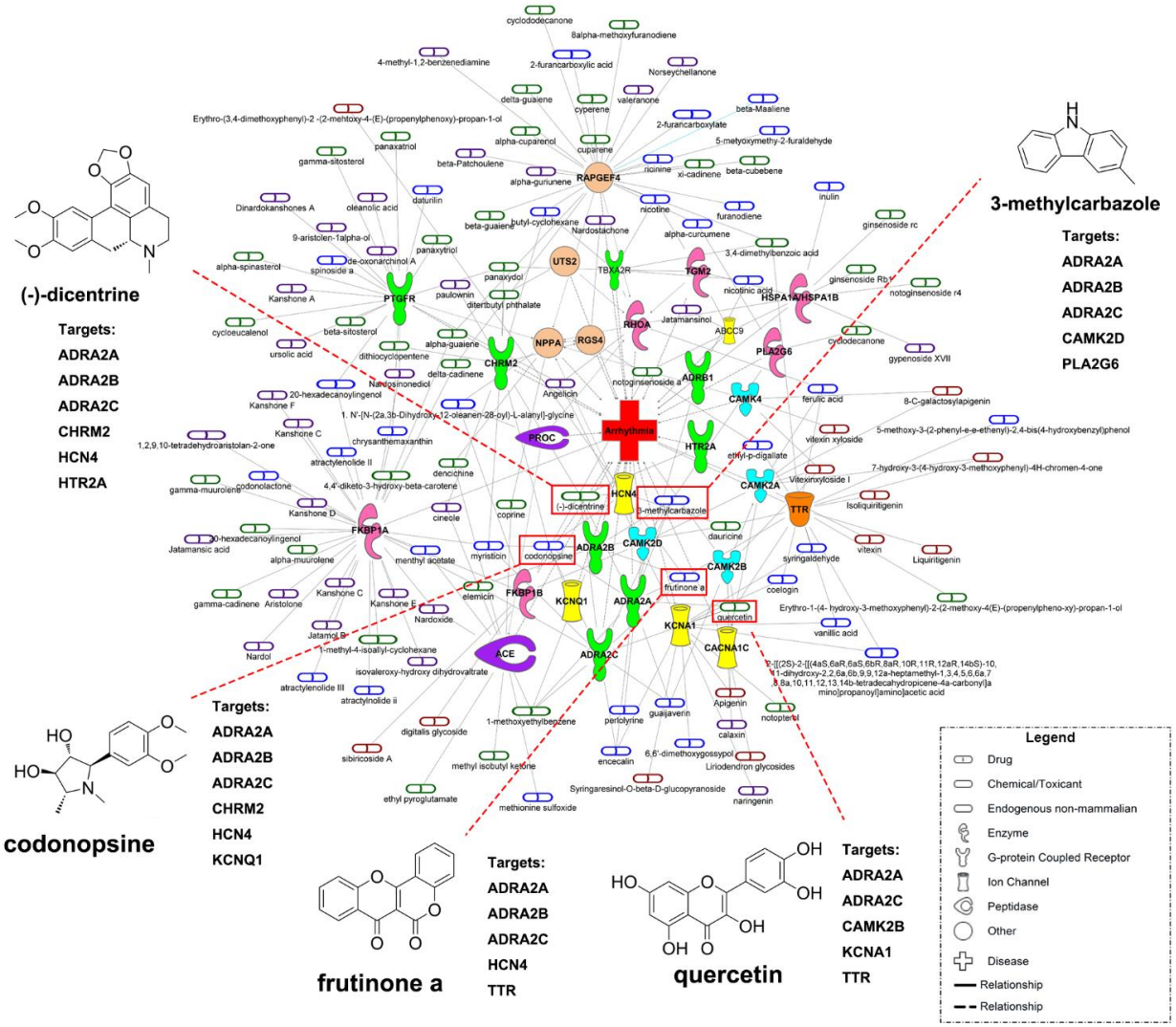
Fig. 5 A disease target network of arrhythmia. The network above showed 153 targets related to arrhythmia were obtained from IPA database as arrhythmic disease target spectrum, all the targets in the network were directly/indirectly related to cardiac arrhythmia. Among the 153 identified targets in the frequency

order, ion channels (in yellow) are 23.78%; enzymes (in pink) are 14.69%; transcription regulators (in blue) are 8.39%; GPCRs (in green) are 7.69%; transporters (in orange) are 6.99%; kinases (in cyan) are 4.20%; growth factors (in grey) are 2.80%; peptidases (in purple) are 2.10%; cytokines (in grey) are 1.40%. Solid line indicated the relationship between the target and arrhythmia was direct , and dotted line indicated the relationship was indirect.

Meanwhile, the common part of disease target spectrum also could be understood as the acting target spectrum of the active components in WK. In total, 216 relationships were predicted with 124 components corresponding to 30 target proteins as the active components in WK (Table S2, ESI†).

Establishment of the molecular pharmacological network of WK

The component target spectrum of WK and arrhythmic disease target spectrum obtained from IPA were connected together, the common part of which was used to build the anti-arrhythmic molecular pharmacological network of WK. In the prediction results, the active components of WK relatively interacted with 30 target proteins: ABCC9, ACE, ADRA2A, ADRA2B, ADRA2C, ADRB1, CACNA1C, CAMK2A, CAMK2B, CAMK2D, CAMK4, CHRM2, FKBP1A, FKBP1B, HCN4, HSPA1A, HTR2A, KCNA1, KCNQ1, NPPA, PLA2G6, PROC, PTGFR, RAPGEF4, RGS4, RHOA, TBXA2R, TGM2, TTR and UTS2, 8 of which are GPCRs, 5 ion channels, 4 kinases, 2 peptidases, 6 enzymes, 1 transporter (Fig. 6).



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346 **Fig. 6** Overall anti-arrhythmic molecular pharmacological network of Wenxin Keli. All
347 the component-target-disease relationships between active components of WK,
348 relevant anti-arrhythmic targets and disease “arrhythmia” were shown in the
349 network. A total of 216 relationships were predicted with 124 components
350 corresponding to 30 target proteins. Part of the components were multi-targets, five
351 of which with most relevant targets were marked with red box and the chemical
352 structures and target spectra were shown as well. Components from Dangshen were
353 marked as blue, Huangjing as red, Sanqi as light green, Hupo as cyan and Gansong as
354 purple.

355

After sub-network analysis, 3 sub-networks were separated from anti-arrhythmic molecular pharmacological network of WK, which are ion channel and internal calcium regulation sub-network, autonomic nervous regulation sub-network, and hormonal regulatory sub-network (Fig. 7A, 7B, 7C). In ion channel and internal calcium regulation sub-network, Dangshen and Gansong contribute 18 nodes relatively, Sanqi 15 nodes and Huangjing 5 nodes, which suggests Dangshen and Gansong showed stronger ability of regulating ion channels (Fig. 7D). However, in autonomic nervous regulation sub-network, Dangshen and Sanqi contributed most and in hormonal regulatory sub-network played the most important role in regulation.

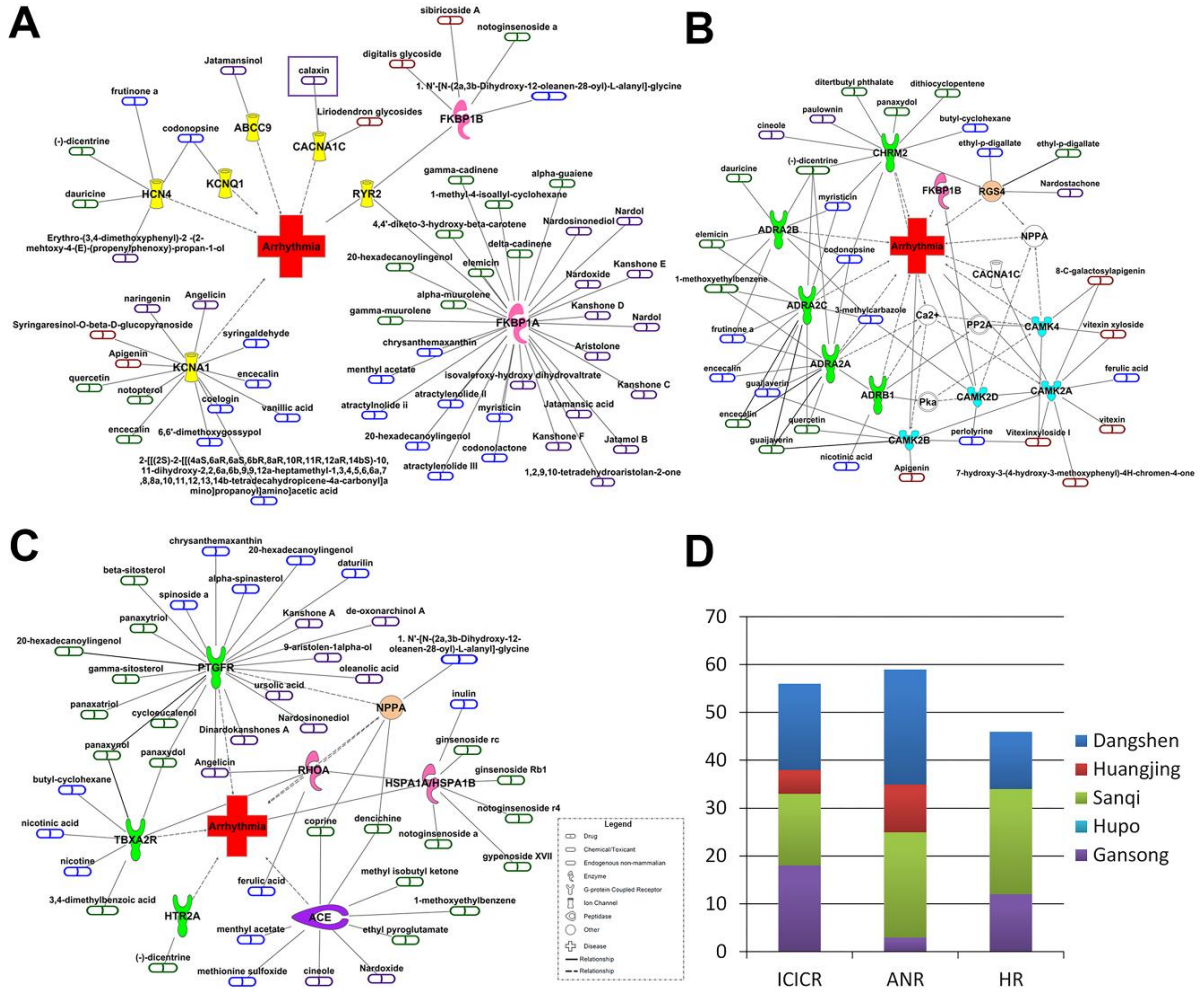

Fig. 7 Major sub-networks of the anti-arrhythmic pharmacological mechanism of Wenxin Keli. **A)** Ion channel and internal calcium regulation network; **B)** autonomic nervous regulation network; **C)** hormonal regulation network; **D)** Contribution of 5

herbs in 3 regulatory sub-networks. The capsule-shape nodes represent chemical components in five herbs of WK, and the others nodes are targets. Components from Dangshen were marked as blue, Huangjing as red, Sanqi as light green, Hupo as cyan and Gansong as purple. Different types of targets were marked by different colors. Directly causal relationship between target and disease were connected by solid line and indirect causal relationship by dashed line. ICICR: Ion channel and internal calcium regulation; ANR: Autonomic nervous regulation; HR: hormonal regulation.

Verification of the electrophysiological effect of WK on Cav1.2 channel

WK was reported to block L-type Calcium channel, but the active component and the subtype of Calcium channel were still uncovered.[64] In our prediction results, one of the components of Gansong, Calaxin, was predicted as a Cav1.2 channel blocker (Fig 8A, marked with purple box), we therefore carried out electrophysiological studies to confirm the curacy of prediction and to uncover part of the anti-arrhythmic mechanism of WK. Results showed that Calaxin can inhibit Cav1.2 channel in a dose dependent manner, the IC50 of which is 120.9 μM (Fig. 8).

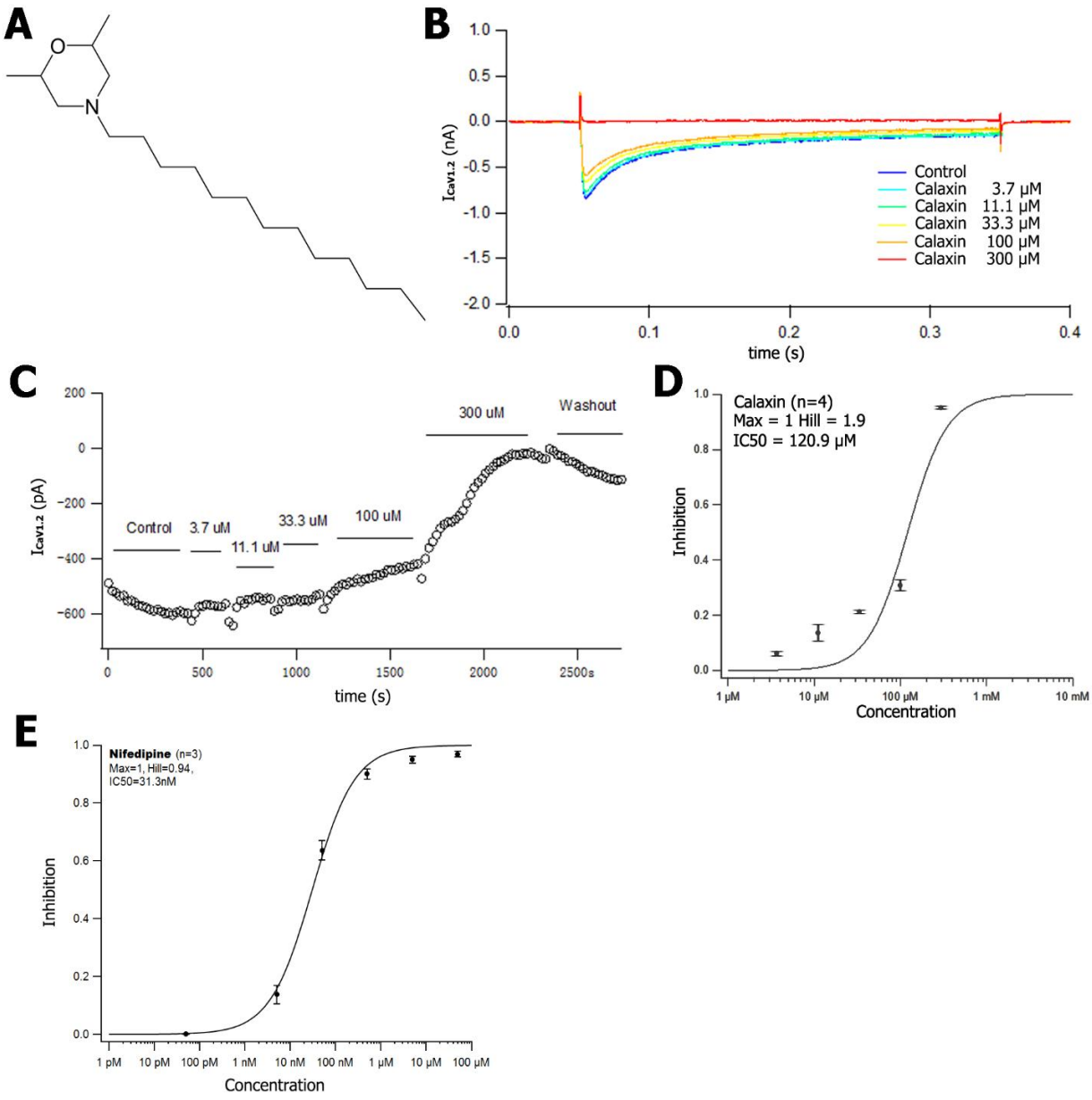

Fig. 8 Inhibitory effect of calaxin on CaV1.2 channel. **A)** Chemical structure of Calaxin; **B)** Cav1.2 current traces inhibited by increasing concentrations of calaxin, plotted against time; **C)** Measurements of Cav1.2 peaks during the application of increasing concentrations of calaxin, plotted against time; **D)** Concentration-response curve for the inhibitory effect of calaxin on CaV1.2 current; **E)** Concentration-response curve for nifedipine, the inhibitory effect of calaxin on CaV1.2 current.

Discussion

In this paper, we successfully established a Naïve Bayesian Model-based method for target prediction of large-scale molecular compounds. It had the ability to predict and rank the possibility of each compound interacting with 1715 target protein simultaneously. We applied this model to carry out target prediction and network analysis for the identified components of 5 herbs/minerals as the representative components of compound Chinese patent drug WK. Using the 124 potential active components with their 30 corresponding protein targets that were obtained, an anti-arrhythmic pharmacological network of WK was established and analysed. Based on these findings, we propose an integrated anti-arrhythmic pharmacological mechanism to explain the protective and therapeutic effect of WK, which includes a quick-acting mechanism regulating ion channels, intracellular calcium and autonomic nervous system, and a long-acting mechanism regulating hormonal regulation system, which also implies that WK may have multiple therapeutic effects with anti-sinus arrhythmia, anti-heart failure and anti-hypertension properties.

Classic anti-arrhythmic drugs, in the stage of drug design and development, tend to interact with (mostly by inhibition) the specific target. Candidate drugs designed under such single-target concept often are strong in pharmacological activity, but with risks of breaking the stability of intracellular physiological system. Accordingly, , a large numbers of anti-arrhythmic drugs developed so far have also been reported to be arrhythmogenic, confirming the deficiency of single-targeted drugs.

In contrast to the "one gene, one drug, one disease" approach of western medicine, “holistic” based traditional Chinese medicine often start with mixtures of multiple herbal extracts. For example, compound Chinese patent medicine generally consists of two or more herbs, the composition complexity of which is much higher than Western medicine. Therefore, for pharmacological studies of traditional Chinese medicine, the primary issue is to model the complex system to reduce the complexity, which will also be more conducive to the understanding and interpretation of the mechanism of traditional Chinese medicine. In this paper, we applied machine-learning approach to establish and optimize target prediction

model with large amounts of bioactivity data to predict the target spectrum of the components of five herbs in WK, and mapped spectrums of the component targets to arrhythmic target obtained from IPA database to establish the anti-arrhythmic molecular pharmacological network of WK.

Traditional Chinese medicine theory suggested that in WK, each of the five herbal/mineral components contributes uniquely to the overall activity of the formula where Gansong acts as a Monarch, Huangjing acts as a Minister, Sanqi and Hupo are Assistants while Gansong serves as a Guide. Our machine-learning based prediction has identified chemical components from Dangshen, Sanqi, Huangjing and Gansong, but not from Hupo, that are capable to interacting with anti-arrhythmic targets. Two possible explanations are: 1. Components in Hupo may not be directly associated with the anti-arrhythmic effects, but play the role of restoring physiological balance by some unknown regulatory mechanisms, and 2. The available chemical database are biased and active components of Hupo has yet to be discovered (Fig. 5). Interestingly, components from the 5 herbs that showed higher connectivity (5 and above), such as (-)-dicentrine, 3-methylcarbazole, codonopsine, frutinone a, and quercetin, also participated in the regulation of more than one type of targets, indicating a greater potential as multi-target anti-arrhythmia agents (Fig. 5).

Sub-network analysis revealed 3 main regulatory mechanisms that may play dominant roles in the anti-arrhythmic effect of WK: First, ion channels and internal calcium system are directly involved in the formation of action potential of heart. Secondly, autonomic nervous system regulates the increase and reduction of heart rate. Finally, hormonal regulation may serve as a sustained or complementary mechanism to the other two.

Besides peak sodium channel, Quinidine also blocks the slowly inactivating, TTX-sensitive sodium current, the slow inward calcium current (ICa), the rapid and slow components of the delayed potassium rectifier current (IKr and IKs), the ATP-sensitive potassium channel (IK, ATP), the inward potassium rectifier current (IK1) and Ito.[65-70] That the overall inhibitory effect of quinidine showed the tendency of

inhibiting outward current during phase 2 and 3 leads to an extended platform of action potential and prolonged Q-T interval. In contrast, WK blocks L-type calcium channel as predicted (Cav1.2, CACNA1C) and supported by Hou's experimental results, which is the main inward current during phase 2.[64] The rebalance of inward and outward currents may explain the anti-arrhythmogenesis effect of WK, meanwhile the other regulatory mechanisms should also be important in stabilized the heart rhythm.

Ion channel and internal calcium regulatory mechanism

Ion channels on cellular cytoplasmic and sarcoplasmic reticulum membrane play a direct and crucial role in the formation of heart rhythm, of which the regular open and close process leads to the generation of action potential. Once the dynamic equilibrium is broken, arrhythmias may occur; correspondingly, most of the anti-arrhythmic drugs have such capacity of rebalancing the ion channels.

In our prediction results, the components in WK interact with five ion channels and two regulators of Ryanodine Receptor 2 (RyR2, coded by gene RYR2), which may be an explanation of quick-acting effect of terminating arrhythmia by WK. In 2013 a meta-analysis research results on WK showed that comparing with western medicine alone, WK was more effective in maintenance of sinus rhythm in patients with paroxysmal atrial fibrillation.[71] The funny current (If, mainly encoded by HCN4) is the leading pacemaker ion channel in the sino-atrial node (SAN). Its specific blocker, e.g. ivabradine, can lower the heart rate selectively.[72] L-type calcium channel Cav1.2 (pore subunit of which is encoded by CACNA1C) is one of the 5 main pacemaker currents in SAN, and also highly expressed in the atrium and ventricle.[73] In 2013, Chen et al discovered that 5g/L WK regulated the L-type Ca2+ channels to treat cardiac hypertrophy and cardiac arrhythmias, at the same time, similar conclusions were drawn by Wang et al, which could be strong experimental evidences to support our prediction results.[13, 74] Cav1.2 is the main channel current of L-type Calcium channel, therefore, Calaxin, one of compounds predicted, was selected as the effective component of WK to confirm the inhibitory effect and partially explain the

chemical substance with anti-arrhythmic property existing in the antiarrhythmic multi-herbal medicine WK.

Kv7.1 play a very important role in cardiac repolarization phase 2 and 3, studies have shown that mutations in KCNQ1 are related to long QT syndrome type 1.[75, 76] Computer simulation experiments confirmed that reduced IKs channel expression leads to ventricular action potential duration significantly prolonged.[77] The component codonopsine was predicted to interact with Kv7.1 which means WK inhibiting potassium channel current is also probably part of its action mechanism.

Intracellular calcium system is a complicated and multifunctional mechanism in regulating heart rate/rhythm, myocardial contractility and some calcium-dependent signal transduction pathways as an intracellular messengers.[78, 79] FKBP12.6 (encoded by FKBP1B) is one of the main regulators interacting with RyR2, which is the major mediator for sarcoplasmic release of stored calcium ions. 4 components (such as notoginsenoside a) showed interaction with FKBP12.6 suggested WK might have the ability of regulating intracellular calcium release.

Ion channel and internal calcium regulatory mechanism of WK can be an explanation of a quick-acting anti-arrhythmic effect of WK as other anti-arrhythmic chemical drugs. Considering the arrhythmogenic effect of Quinidine, the irrational multi-target regulation may be an essential reason. Besides peak sodium channel, Quinidine also blocks the slowly inactivating, TTX-sensitive sodium current, the slow inward calcium current (ICa), the rapid and slow components of the delayed potassium rectifier current (IKr and IKs), the ATP-sensitive potassium channel (IK, ATP), the inward potassium rectifier current (IK1) and Ito. That the overall inhibitory effect of quinidine showed the tendency of inhibiting outward current during phase 2 and 3 leads to an extended platform of action potential and prolonged Q-T interval. The protective effect of WK against the arrhythmogenic effect may act as rebalancing the inward and outward currents to keep the parameters of ECG in the normal range.

Autonomic nervous regulatory mechanism

In human heart, cardiac function is under the regulation of the sympathetic and parasympathetic nervous system. Autonomic nervous system plays an important role in the pathophysiology of arrhythmia, at present α2-adrenoceptor agonist, β1-adrenoceptor blocker and HCN inhibitor are most widely used clinical strategies to reduce sympathetic tone.[80]

β-Adrenergic receptor (β-AR) activation can provoke cardiac arrhythmias mediated by cAMP-dependent Ca2+ release from the sarcoplasmic reticulum (SR) via phosphorylation of the RyR2. However, cAMP can activate both protein kinase A and an exchange protein directly activated by cAMP (Epac). Epac2 mediates β1-AR–induced cardiac arrhythmias via CaMKII and RyR2-S2814 phosphorylation. M2 muscarinic receptor (M2-AchR, encoded by gene CHRM2) is the representative target of cholinomimetic compounds, which regulates the activity of pacemaker in sinoatrial node (SAN), atrioventricular conducting velocity and the force of myocardial contraction.[81-84] Activation of M2-AchR coupled with pertussis toxin-sensitive G protein (Gi) leads to the inhibition of adenylylcyclase, which causes a series of changes in downstream signal pathways.[85, 86] 18 components showed β1-AR , M2-AchR and CaMKII regulatory activity, suggest that WK might inhibits cardiac arrhythmias by regulating the autonomic nervous system. Interestingly, the regulatory effect of CaMKII by WK has been confirmed by Xing et al in 2013.[15]

α-adrenergic receptor not only directly excites cardiomyocytes, but also directly affect platelet aggregation and the coronary hemodynamics function.[87, 88] Activation or blocking of α-adrenergic receptors does not affect the normal cardiac electrical stability, however, in the case of myocardial ischemia, α-adrenergic receptor blockers can reduce coronary contraction, reduce platelet aggregation.[89, 90] 3 subtypes of α2-adrenoceptors were confirmed by gene cloning: α2A, α2B and α2C, studies in mouse revealed that both the α2A and α2C subtypes were required for normal presynaptic control of transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons, whereas the α2B subtype modulated the vasoconstriction.[91-94] Regulation of α2-adrenoceptors by WK may be a secondary way to treat arrhythmia, but it still needs further experimental evidence.

In summary, WK perhaps has such efficacy of regulating heart rate by interacting with α2-adrenoceptors, β1-AR, M2-AchR and the proteins in these downstream pathways to regulate the autonomic nervous system to relieve cardiac arrhythmia.

Hormonal regulatory mechanism

Studies showed that endogenous prostaglandin compounds in vivo in rats are able to cause a biphasic increase in heart rate by interacting with PTGFR and TBXA2R receptors, while blocking or knocking out these two receptors, the increase of heart rate will be significantly inhibited.[95] In the five herbs of which WK is composed, 20 components were predicted to interact with PTGFR, TBXA2R, suggesting WK has a high possibility of blocking prostaglandin to lower the high heart rate.

In 1995, researchers found that atrial fibrillation can trigger a series of changes in the electrical characteristics of the heart, leading to persistent atrial fibrillation.[96] Recent studies have shown that angiotensin-converting enzyme (ACE) blockers can block atrial electrical remodelling at the beginning of this process, and electrical remodelling occurs earlier than the occurrence of atrial fibrillation [97, 98] ACE is one of the key enzymes in renin-angiotensin system, which catalyzes angiotensin I converting to angiotensin II. Renin-angiotensin system plays important role in myocardial fibrosis caused by heart failure, experiments revealed that atrial fibrosis and cardiac conduction abnormalities relieved in Enalapril (an ACE inhibitor) pre-treated canine model, furthermore, the incidence of atrial fibrillation in Enalapril treated patients with left ventricular dysfunction also decreased.[99-101] 10 components in WK were predicted to interact with ACE suggests that blocking ACE may be one of the anti-arrhythmic mechanism of WK. In 2013, Wu et al found that WK relieves ventricular remodelling and myocardial apoptosis in rats with myocardial infarction by reducing angiotensin II concentration, which indirectly supported our prediction results.[102]

Hormonal regulation mechanism of WK suggests that there may be such a long-acting regulatory mechanism which protects the heart from arrhythmia or failure through an indirect way but not related to ion channels.

Conclusion

We can draw the following conclusions from the present study: (1) WK effectively suppresses the arrhythmogenesis by high concentration of quinidine, alleviate the prolongation of QT interval by quinidine, and sustains the normal shape of ECG. (2) The integrative pharmacological mechanism on anti-arrhythmic and protective effect of WK consists of 124 components corresponding to 30 target proteins, which covers 3 main regulations: ion channel & internal calcium regulation, autonomic nervous and hormonal regulation. (3) One of the components above, Calaxin, was proved blocking Cav1.2 channel through electrophysiological assay.

Acknowledgements

This work was supported by grants from the International Cooperation Project of MOST, China (2013DFA31620), the National Major New Drug Discovery (2013ZX0920102) and the National Science Foundation of China (NSFC 81274128). We thank our colleagues Drs. Ying Cui, Yuefei Wang, Honghua Wu, Guixiang Pan, Yantong Xu, and Yu Wang for stimulating discussions and technical supports. The authors report no conflicts of interest in this work.

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