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Regular Article

Preclinical evaluation of marketed sodium channel blockers in a rat model of myotonia discloses promising antimyotonic drugs

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ABSTRACT

Although the sodium channel blocker mexiletine is considered the first-line drug in myotonia, some patients experiment adverse effects, while others do not gain any benefit. Other antimyotonic drugs are thus needed to offer mexiletine alternatives. In the present study, we used a previously-validated rat model of myotonia congenita to compare six marketed sodium channel blockers to mexiletine. Myotonia was induced in the rat by injection of anthracen-9-carboxylic acid, a muscle chloride channel blocker. The drugs were given orally and myotonia was evaluated by measuring the time of righting reflex. The drugs were also tested on sodium currents recorded in a cell line transfected with the human skeletal muscle sodium channel hNav1.4 using patch-clamp technique. In vivo, carbamazepine and propafenone showed antimyotonic activity at doses similar to mexiletine (ED_{50} close to 5 mg/kg); flecainide and orphenadrine showed greater potency (ED_{50} near 1 mg/kg); lubeluzole and riluzole were the more potent (ED_{50} near 0.1 mg/kg). The antimyotonic activity of drugs in vivo was linearly correlated with their potency in blocking hNav1.4 channels in vitro. Deviation was observed for propafenone and carbamazepine, likely due to pharmacokinetics and multiple targets. The comparison of the antimyotonic dose calculated in rats with the current clinical dose in humans strongly suggests that all the tested drugs may be used safely for the treatment of human myotonia. Considering the limits of mexiletine tolerability and the occurrence of non-responders, this study proposes an arsenal of alternative drugs, which may prove useful to increase the quality of life of individuals suffering from non-dystrophic myotonia. Further clinical trials are warranted to confirm these results.

Introduction

Myotonic disorders are characterized by skeletal muscle stiffness after voluntary contraction or percussion. Inherited myotonias include dystrophic and non-dystrophic diseases and can be subdivided in various disease entities on the basis of clinical phenotype and genotype (Matthews et al., 2010; Rayan and Hanna, 2010; Trivedi et al., 2013b). Non-dystrophic myotonias (NDM) are caused by mutations in genes encoding the voltage-dependent chloride channel (*CLCN1* gene, ClC-1 protein) or sodium channel (*SCN4A* gene, Nav1.4 protein), which are expressed exclusively in skeletal muscles. The reduced activity of mutated ClC-1 channels or increased activity of mutated Nav1.4 channels determine a pathological sarcolemma hyperexcitability with occurrence of high-frequency action potential discharges; the consequent difficulty in muscle relaxation is responsible for the characteristic stiffness of myotonic muscle. NDM can be chronically debilitating due to pain and muscle stiffness, which are associated with frequent falls and disability. In the more severe cases, myotonia can prohibit sport practice, limit ability at school, and alter physical growth.

Pharmacotherapy is often needed for myotonic patients. Today, the class IB antiarrhythmic, mexiletine, is considered as the drug of choice. By blocking skeletal muscle sodium channels, mexiletine can contrast high frequency firing of myotonic action potentials. Recent clinical trials have confirmed the usefulness of mexiletine in both dystrophic and non-dystrophic myotonias, irrespective of the culprit gene (Logigian et al., 2010; Statland et al., 2012). Consequently, mexiletine was recently appointed by FDA and EMA as an orphan drug in NDM. Mexiletine preferentially binds the open and/or inactivated sodium channels, resulting in a use-dependent block that is thought to constitute the basis of the selective action of mexiletine on pathologic hyperactive tissues (Conte Camerino et al., 2007; Desaphy et al., 1999; Wang et al., 2004).

Nevertheless not all the myotonic individuals obtain benefits from mexiletine, because the drug can induce side effects limiting patient compliance (mainly epigastric discomfort, nausea, tremor, anxiety, dizziness, lightheadedness, and headaches). Particular attention must be also paid to cardiomyopathic patients, who may be exposed to life-threatening complications. In addition, non responders to

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mexiletine therapy have been occasionally observed, likely due to pharmacogenetic mechanisms (Desaphy et al., 2001, 2013a, 2013b, 2013c). Last but not least, mexiletine was withdrawn from the market in several countries, leaving patients and doctors with an unmet medical need. It is thus likely that a number of myotonic individuals would obtain significant improvement of quality of life from the individuation of new efficient and safe antimyotonic drugs.

Other sodium channel blockers have been anecdotally reported to exert antimyotonic activity, including carbamazepine, flecainide, and propafenone, although no randomized clinical trial has been performed in NDM (Alfonsi et al., 2007; Desaphy et al., 2013a; Lyons et al., 2010; Rosenfeld et al., 1997; Savitha et al., 2006; Sechi et al., 1983). Flecainide and propafenone resulted efficient in patients with specific sodium channel mutations, who were resistant to mexiletine (Alfonsi et al., 2007; Desaphy et al., 2013a; Rosenfeld et al., 1997). Nevertheless, there is no available information regarding the relative efficiency of these drugs in the whole myotonic population. It is worth to note that no chloride channel openers are available.

Starting from these considerations, we have recently developed a preclinical rat model of myotonia in vivo to allow drug screening (Desaphy et al., 2013b). In this model, inhibition of the ClC-1 chloride channel by anthracen-9-carboxylic acid (9AC) induces muscle stiffness that increases the time of righting reflex (TRR) of the rat. Thus we were able to quantitatively assess the in vivo antimyotonic activity of orally-administrated mexiletine and β-adrenergic drugs that also block sodium channels in a use-dependent manner (Desaphy et al., 2003, 2013b).

In the present study, we used this preclinical model to evaluate in vivo the antimyotonic activity of a number of marketed sodium channel blockers. The tested drugs were chosen because they were previously reported to relieve myotonia in humans (mexiletine, carbamazepine, flecainide, and propafenone) (Alfonsi et al., 2007; Desaphy et al., 2013a; Lyons et al., 2010; Rosenfeld et al., 1997; Savitha et al., 2006; Sechi et al., 1983), and/or to exert significant block of human skeletal muscle sodium channels in cell lines (orphenadrine, lubeluzole, and riluzole) (Desaphy et al., 2001, 2004, 2009, 2012, 2013c). Patch clamp experiments were performed to verify the correlation between use-dependent block of heterologously-expressed hNav1.4 channels and antimyotonic efficiency in vivo.

Materials and methods

Animal care and in vivo experiments

The experiments were performed in accordance with the Italian Guidelines for the use of laboratory animals, which conforms to the European Union Directive for the protection of experimental animals (2011/63/EU), and received approval from the Animal Experimentation Ethic Committee of the University of Bari (CESA prot. 7/12) and Italian Health Department (Decreto n. 91/2013-B). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Adult WISTAR rats (350–500 g) purchased from Charles River—Italy were housed individually and given food and water ad libitum. In a typical daily experiment, three/four rats were randomly assigned to receive an intraperitoneal injection of 30 mg/kg anthracen-9-carboxylic acid (9AC), as previously described (Desaphy et al., 2013b). Ten minutes after 9-AC injection, the animals were given drug or vehicle per os using an esophageal cannula. Myotonia state was assayed by measuring the time of righting reflex (TRR), that is the time taken by the rat to turn back on his four limbs after having been positioned in supine position. Ambient temperature was maintained to 20–22 °C. The TRR was determined 10 min before and 10, 30, 60, 120, and 180 min after 9AC administration. At each time point, the TRR was calculated as the average of 7 trials, which allows obtaining a S.E.M. minor than 10% of the mean. A 1-minute interval was respected between two trials to avoid any warm-up phenomenon. Typically, a control rat takes less than 0.5 s to turn back on his limbs, whereas, 30 min after 9AC injection, a myotonic rat shows a TRR greater than 3 s. An antimyotonic drug is expected to reduce the TRR with respect to vehicle. Effect of 9AC was maximal 30 min after injection and reversed spontaneously within 15 h. At the end of each experimental day, the tested rats were allowed a resting period of at least 48 h before to reintegrate the reserve pool. A number of experiments have been performed blindly, meaning that the two operators who measured the TRR in the four rats in the daily experiment were not aware of the drug dose given to each rat. No apparent difference was found between blind and open experiments.

This protocol was used to evaluate the time course of drug effects at each dose. For each drug dose, the protocols were repeated on three-to-four days in different rats, and the experimental points are given as the mean ± S.E.M. Statistical analysis was performed by ANOVA followed by ad-hoc Bonferroni's *t*-test to compare differences between drug doses at each time point. Differences were considered significant with $P < 0.05$. The dose–response relationships were drawn by reporting the mean TRR measured at each drug dose normalized with respect to the mean TRR measured with drug vehicle as a function of the drug dose. The relationships were fit with equation reported in figure legend.

Sodium current measurement in HEK293 cells

Whole-cell sodium currents (I_{Na}) were recorded with patch-clamp technique in HEK293 cells permanently transfected with the human skeletal muscle isoform of voltage-gated sodium channel, hNav1.4 (Desaphy et al., 2012). Sodium current recordings were performed at room temperature (20–22 °C) using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Voltage clamp protocols and data acquisition were performed with pCLAMP 9.2 software (Axon Instruments) through a 12-bit A-D/D-A interface (Digidata 1340, Axon Instruments). Pipettes made with Corning 7052 glass (Garner Glass, Claremont, CA, USA) had resistance that ranged from 1 to 3 MΩ. Currents were low-pass filtered at 2 kHz (−3 dB) by the four-pole Bessel filter of the amplifier and digitized at 10–20 kHz. After the rupture of patch membrane, a test pulse of −30 mV from a holding potential of −120 mV was applied to the cell until stabilization of sodium current amplitude and kinetics (typically 5 min). Only those data obtained from cells exhibiting voltage errors <5 mV after series resistance compensation were considered for analysis. Little (<5%) or no rundown was observed within the experiments.

Drugs and solutions

Patch clamp pipette solution contained in mM: 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA and 5 HEPES, and pH was set to 7.2 with CsOH. Bath solution for patch clamp recordings contained (in mM): 150 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES and 5 glucose. The pH was set to 7.4 with NaOH. All the compounds were purchased from Sigma-Aldrich (Milan, Italy).

Mexiletine hydrochloride, anthracen-9-carboxylic acid (9AC), carbamazepine, carbamazepine 10,11-epoxide (epo-CBZ), propafenone hydrochloride, flecainide acetate, orphenadrine hydrochloride, were purchased from Sigma-Aldrich (Milan, Italy). Lubeluzole (R,S racemate) and riluzole were synthesized in the medicinal chemistry laboratories of our department as previously described (Bruno et al., 2006; Catalano et al., 2013; Desaphy et al., 2013c).

For patch-clamp experiments, the drugs were dissolved at the desired final concentration in external patch solution containing up to 1% dimethylsulfoxide. In our experimental conditions, we found no effect of 1% DMSO on sodium channels. Notwithstanding, control recordings were made in cells bathed with drug-free solutions containing the same amount of DMSO. The patched cell was continuously exposed to a stream of control (supplemented with DMSO as needed) or drug-