

## Effects of natural and synthetic isothiocyanate-based H<sub>2</sub>S-releasers against chemotherapy-induced neuropathic pain: Role of Kv7 potassium channels



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### ABSTRACT

Hydrogen sulfide (H<sub>2</sub>S) is a crucial signaling molecule involved in several physiological and pathological processes. Nonetheless, the role of this gasotransmitter in the pathogenesis and treatment of neuropathic pain is controversial.

The aim of the present study was to investigate the pain relieving profile of a series of slow releasing H<sub>2</sub>S donors (the natural allyl-isothiocyanate and the synthetics phenyl- and carboxyphenyl-isothiocyanate) in animal models of neuropathic pain induced by paclitaxel or oxaliplatin, anticancer drugs characterized by a dose-limiting neurotoxicity. The potential contribution of Kv7 potassium channels modulation was also studied.

Mice were treated with paclitaxel (2.0 mg kg<sup>-1</sup>) i.p. on days 1, 3, 5 and 7; oxaliplatin (2.4 mg kg<sup>-1</sup>) was administered i.p. on days 1–2, 5–9, 12–14. Behavioral tests were performed on day 15. In both models, single subcutaneous administrations of H<sub>2</sub>S donors (1.33, 4.43, 13.31 μmol kg<sup>-1</sup>) reduced the hypersensitivity to cold non-noxious stimuli (allodynia-related measurement). The prototypical H<sub>2</sub>S donor NaHS was also effective. Activity was maintained after i.c.v. administrations. On the contrary, the S-lacking molecule allyl-isocyanate did not increase pain threshold; the H<sub>2</sub>S-binding molecule hemoglobin abolished the pain-relieving effects of isothiocyanates and NaHS. The anti-neuropathic properties of H<sub>2</sub>S donors were reverted by the Kv7 potassium channel blocker XE991. Currents carried by Kv7.2 homomers and Kv7.2/Kv7.3 heteromers expressed in CHO cells were potentiated by H<sub>2</sub>S donors.

Systemically- or centrally-administered isothiocyanates reduced chemotherapy-induced neuropathic pain by releasing H<sub>2</sub>S. Activation of Kv7 channels largely mediate the anti-neuropathic effect.

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**Abbreviations:** A-ITC, allyl isothiocyanate; A-IC, allyl isocyanate; CAT, cysteine aminotransferase; CBS, cystathionine-β-synthase; CMC, carboxymethyl cellulose; CO, carbon monoxide; CP-ITC, 3-carboxyphenyl isothiocyanate; CSE, cystathionine-γ-lyase; Hb, hemoglobin; H<sub>2</sub>S, hydrogen sulphide; 3MST, 3-mercaptopyruvate sulfurtransferase; NaHS, sodium hydrosulfide hydrate; NO, nitric oxide; PEG, polyethylene glycol; P-ITC, phenyl isothiocyanate; TEA, tetraethylammonium.

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## 1. Introduction

H<sub>2</sub>S is historically known as a natural chemical hazard. Nowadays H<sub>2</sub>S is considered a gasotransmitter in the mammalian body, like NO and CO, contributing to many physiological and pathological processes (Wang, 2002). It is endogenously produced mostly from L-cysteine and homocysteine through CSE, CBS and by a third pathway catalyzed by 3MST along with CAT (Shibuya et al., 2009). H<sub>2</sub>S can also be generated by sulfate-reducing bacteria in the intestinal lumen (Roediger et al., 1997). CBS is preferentially

expressed in the hippocampus and cerebellum (Abe and Kimura, 1996), mainly localized in astrocytes and microglial cells (Enokido et al., 2005). Despite the high level of CSE in the cardiovascular system, the enzyme was also found in the nervous tissue in microglial cells (Lee et al., 2006) and in spinal cord- (Distrutti et al., 2006) and cerebellar granule-neurons (Garcia-Bereguain et al., 2008). Also 3MST is present in neurons and astrocytes (Shibuya et al., 2009; Zhao et al., 2013).

In the nervous system, H<sub>2</sub>S is involved in synaptic modulation by interacting with ion channels, second messengers and modifying sulfhydryl groups of proteins (Wallace and Wang, 2015). A major role in redox balance, mitochondrial bioenergetics, apoptosis and inflammatory processes (Kamat et al., 2015) leads to H<sub>2</sub>S relevance in memory, cognition and learning (Nagpure and Bian, 2015) as well as in neuroprotection (Paul and Snyder, 2015).

The role of H<sub>2</sub>S in pain modulation is controversial: conflicting data suggest that it is both pronociceptive (mainly through Cav3.2 T-type Ca<sup>2+</sup> channels and transient receptor potential ankyrin 1 – TRPA1 channels; Terada and Kawabata, 2015) and antinociceptive (Wallace and Wang, 2015) depending on the models used and the types and doses of H<sub>2</sub>S donors or modulators used. Nonetheless, several studies have suggested significant pain relieving effects of H<sub>2</sub>S donors against neuropathic and visceral pain (Distrutti et al., 2006; Lin et al., 2014; Kida et al., 2015). Recently, some isothiocyanate derivatives, slow releasing H<sub>2</sub>S-donor moieties, and NaHS, a prototypical H<sub>2</sub>S-generating agent, were described as activators of Kv7 potassium channels (Testai et al., 2015; Martelli et al., 2013a), a class of the voltage-gated potassium channels which plays a pivotal role in pain modulation (Zheng et al., 2013; Busserolles et al., 2016). A decreased expression of Kv7 channels contributes to neuropathic hyperalgesia (Rose et al., 2011) since channel activation inhibits C and A $\delta$  fiber-mediated responses of dorsal horn neurons (Passmore et al., 2003) and reduces the ectopic generation of action potentials in neuropathic pain (Lang et al., 2008). On these premises, Kv7 channel activators like retigabine are currently regarded as anti-hyperalgesic compounds (Nodera et al., 2011; Blackburn-Munro and Jensen, 2003).

Aim of the present work was to investigate the pharmacological profile of different isothiocyanates (the chemical structures are depicted in Scheme 1), after acute subcutaneous administration in mouse models of chemotherapy-induced neuropathic pain evoked by paclitaxel and oxaliplatin (Di Cesare Mannelli et al., 2013; Fariello et al., 2014). In particular, allyl isothiocyanate, naturally occurring in many species of Brassicaceae (Citi et al., 2014), and the synthetic phenyl- and carboxyphenyl isothiocyanate were

compared with NaHS. The pharmacodynamic involvement of H<sub>2</sub>S and Kv7 was studied.

## 2. Methods

### 2.1. Animals

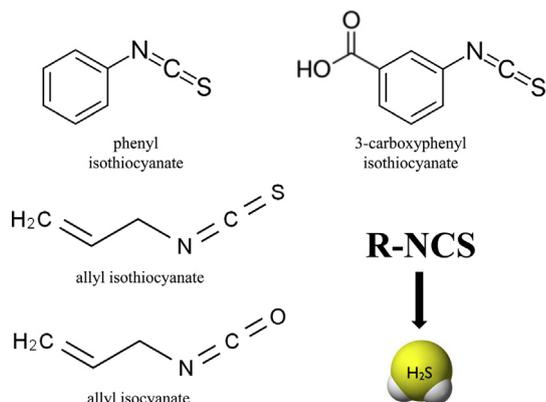
Male CD-1 albino mice (Envigo, Varese, Italy) weighing approximately 22–25 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Ten mice were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Paclitaxel- and oxaliplatin-induced neuropathic pain models

Mice treated with paclitaxel (2.0 mg kg<sup>-1</sup>) were injected i.p. on four alternate days (days 1, 3, 5 and 7) (Polomano et al., 2001). Paclitaxel was dissolved in a mixture of 10% saline solution and Cremophor EL, a derivative of castor oil and ethylene oxide that is clinically used as paclitaxel vehicle. Mice treated with oxaliplatin (2.4 mg kg<sup>-1</sup>) were administered i.p. on days 1–2, 5–9, 12–14 (10 i.p. injections) (Cavaletti et al., 2001). Oxaliplatin was dissolved in 5% glucose solution. Control animals received an equivalent volume of vehicle. Behavioral tests were performed on day 15.

### 2.3. Compound administrations and in vitro uses

A-ITC, A-IC, P-ITC (Sigma-Aldrich, Milan, Italy) and CP-ITC (Fluorochem Ltd, Hadfield, UK) were dissolved in saline solution with 0.5% PEG. GYY4137 dichloromethane complex and NaHS (Sigma-Aldrich, Milan, Italy) were dissolved in saline solution. Morphine (S.A.L.A.R.S., Como, Italy) and pregabalin (3B Scientific Corporation, China) were dissolved in saline solution. Celecoxib (Sigma-Aldrich, Italy) and duloxetine (Sequoia Research, UK) were dissolved in 1% CMC. Compounds were acutely administered as follows. The doses of NaHS were chosen on the bases of previously published H<sub>2</sub>S releasing and antinociceptive properties (Distrutti et al., 2006; Martelli et al., 2013b) (1.33, 4.43, 13.31 and 38 μmol kg<sup>-1</sup> corresponding to 0.1, 0.33, 1 and 3 mg kg<sup>-1</sup> were administered by s.c. route or 4.43 nmol/mouse by i.c.v. route); P-ITC (1.33, 4.43 and 13.31 μmol kg<sup>-1</sup> s.c. or 4.43 nmol/mouse i.c.v.); CP-ITC, A-ITC and A-IC (1.33, 4.43 and 13.31 μmol kg<sup>-1</sup> s.c.); GYY4137 (4.43 μmol kg<sup>-1</sup> s.c.). As reference compounds were used morphine (21.75 μmol kg<sup>-1</sup>; 7 mg kg<sup>-1</sup> s.c.; Rashid et al., 2004), pregabalin (94.20 μmol kg<sup>-1</sup>; 15 mg kg<sup>-1</sup> s.c.; Di Cesare Mannelli et al., 2015a), celecoxib (104.88 μmol kg<sup>-1</sup>; 40 mg kg<sup>-1</sup> p.o.; Jiang et al., 2016) and duloxetine (50.43 μmol kg<sup>-1</sup>; 15 mg kg<sup>-1</sup> p.o.; Di Cesare Mannelli et al., 2015a). In additional experiment P-ITC, NaHS and GYY4137 (4.43 μmol kg<sup>-1</sup>) were administered s.c. in mixture with 3.10 μmol kg<sup>-1</sup> (200 mg kg<sup>-1</sup>) human Hb (Sigma-Aldrich, Italy) in



**Scheme 1.** Chemical structures of tested compounds and representation of H<sub>2</sub>S release.

saline solution and behavioral tests were carried out after 15, 30, 45 and 60 min from injection. The Kv7 potassium channel blocker XE991 (Tocris Bioscience, Italy;  $2.66 \mu\text{mol kg}^{-1}$ ;  $1 \text{ mg kg}^{-1}$ ; Blackburn-Munro and Jensen, 2003) was dissolved in saline solution and administered i.p. 15 min before the injection of tested compounds. The Kv7 potassium channel opener retigabine (Sequoia Research, UK;  $65.93 \mu\text{mol kg}^{-1}$ ;  $20 \text{ mg kg}^{-1}$ ; Blackburn-Munro and Jensen, 2003; Miceli et al., 2008) was dissolved in 1% CMC and administered p.o.

In electrophysiological measurements, retigabine (Valeant Pharmaceuticals, USA) and CP-ITC were dissolved in DMSO, whereas NaHS (Sigma-Aldrich, Italy) was dissolved in water. In each experiment, the same volume of solvent present in the drug solution was added to the control solution; maximal concentration of DMSO (0.1%) was *per se* ineffective. A-ITC, A-IC and P-ITC are liquid at room temperature, thus requiring no solvent addition in the respective control solution. Fresh drug stocks (at M concentration) were prepared daily or, as in the case of NaHS, twice daily. Fast solution exchanges ( $<1 \text{ s}$ ) were achieved, as previously reported (Ambrosino et al., 2015), by means of a cFlow 8 flow controller attached to a cF-8VS 8-valve switching apparatus and a MPRE8 miniature flow outlet having an inside diameter of  $360 \mu\text{m}$  and a mixing volume at the tip of  $1\text{--}2 \mu\text{l}$  (Cell Microcontrols, Norfolk, VA, USA) positioned within  $200 \mu\text{m}$  of the cell.

#### 2.4. Cold plate test

The animals were placed in a stainless steel box ( $12 \text{ cm} \times 20 \text{ cm} \times 10 \text{ cm}$ ) with a cold plate as floor. The temperature of the cold plate was kept constant at  $4 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ . Pain-related behavior (licking of the hind paw) was observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s (Di Cesare Mannelli et al., 2013).

#### 2.5. Von Frey test

Animals were placed in  $10 \text{ cm} \times 10 \text{ cm}$  Plexiglas boxes equipped with a metallic mesh floor, 20 cm above the bench. Animals were allowed to habituate themselves to their environment for 15 min before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying forces ranging from 0 to 5 g with 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each posterior paw from below the mesh floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum force required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not considered as a withdrawal response. Stimuli were applied to each anterior paw at 5 s intervals. Measurements were repeated 5 times and the final value was obtained by averaging the 5 measurements (Ta et al., 2009).

#### 2.6. Hot-plate test

Hot-plate test was carried out accordingly with O'Callaghan and Holzman (1975). Mice were placed inside a stainless steel container, thermostatically set at  $52.5 \pm 0.1 \text{ }^\circ\text{C}$  in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s) were measured with a stop-watch before and at regular intervals up to a maximum of 60 min after treatment. The end point used was the licking of the fore or hind paws. Before treating animals tested compounds, a pretest was performed: those mice scoring below 12 and over 18 s were rejected. An arbitrary cutoff time of 45 s was

adopted.

#### 2.7. Cell culture and transfection

As previously described (Miceli et al., 2013), CHO cells were grown in DMEM medium supplemented with 10% FBS,  $100 \text{ U mL}^{-1}$  penicillin/streptomycin and 2 mM L-glutamine. The cells were kept in a humidified atmosphere at  $37 \text{ }^\circ\text{C}$  with 5%  $\text{CO}_2$  in 100 mm plastic Petri dishes. Twenty-four hours after plating on 35-mm glass coverslips (Carolina Biological Supply Co., Burlington, NC) coated with poly-L-lysine (Sigma, Milan, Italy), CHO cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Milan, Italy), according to the manufacturer protocols, with plasmids containing the cDNAs encoding for Kv7.2 or Kv7.2 and Kv7.3 (cDNA transfection ratio 1:1) and for the Enhanced Green Fluorescent Protein (Clontech, Palo Alto, CA; 1/10 of the total cDNA), used as a transfection marker.

#### 2.8. Patch-clamp electrophysiology

Currents from CHO cells were recorded at room temperature ( $20\text{--}22 \text{ }^\circ\text{C}$ ) 1 day after transfection by using an Axopatch 200A (Molecular Devices) and the whole-cell configuration of the patch-clamp technique, with glass micropipettes of  $3\text{--}5 \text{ M}\Omega$  resistance, as previously described (Ambrosino et al., 2015). The extracellular solution contained (in mM) 138 NaCl, 2  $\text{CaCl}_2$ , 5.4 KCl, 1  $\text{MgCl}_2$ , 10 glucose, and 10 Hepes, pH 7.4 adjusted with NaOH; the pipette (intracellular) solution contained (in mM) 140 KCl, 2  $\text{MgCl}_2$ , 10 EGTA, 10 Hepes, 5 Mg-ATP, pH 7.3 to 7.4 adjusted with KOH. pCLAMP software (version 10.0.2) was used for data acquisition and analysis. Linear cell capacitance (C) was determined by integrating the area under the whole-cell capacity transient, evoked by short ( $5\text{--}10 \text{ ms}$ ) pulses from  $-80$  to  $-75 \text{ mV}$  with the whole-cell capacitance compensation circuit of the Axopatch 200A turned off. Currents were activated by 3-s voltage ramps from  $-80 \text{ mV}$  to 0 mV at 0.1-Hz frequency. Current densities (expressed in pA/pF) were calculated by dividing currents at 0 mV by C. Currents were corrected offline for leakage currents using standard subtraction routines (Clampfit module of pClamp 10). In analogy to the reported effects of the prototypical Kv7 activator retigabine (Miceli et al., 2008), two parameters were used to quantify drug-induced effects: the percent of current potentiation at 0 mV, and the leftward shift of the membrane potential at which currents measured during drug application were identical to those recorded in control solution at  $-40 \text{ mV}$  (Miceli et al., 2013); this latter parameter was arbitrarily defined as  $\Delta V_{-40}$ . Both parameters were determined after 1–2-min drug application, a time sufficient for drug-induced effects to reach steady-state. Current activation and deactivation kinetics were analyzed by fitting the current traces to a single or to a double-exponential function of the following form:  $y = \text{amp}_f \exp(-t/\tau_f) + \text{amp}_s \exp(-t/\tau_s)$ , where  $\text{amp}_f$  and  $\text{amp}_s$  indicate the amplitude of the fast and slow exponential components respectively, whereas  $\tau_f$  and  $\tau_s$  indicate the time constants of these components, as previously described (Soldovieri et al., 2007).

#### 2.9. Statistical analysis

Behavioral measurements were performed on 16 mice for each treatment carried out in 2 different experimental sets. Results were expressed as mean  $\pm$  S.E.M. The analysis of variance of behavioral data was performed by one way ANOVA, a Bonferroni's significant difference procedure was used as post-hoc comparison. *P* values of less than 0.05 or 0.01 were considered significant. Statistically significant differences in electrophysiology data were evaluated with the Student *t*-test, or with ANOVA followed by the Student-

Newman-Keuls test when multiple groups were compared, with the threshold set at  $P < 0.05$ . Investigators were blind to all experimental procedures. Data were analyzed using the “Origin 9” software (OriginLab, Northampton, USA). The area under the curve (AUC) of the antinociceptive effects was calculated using the same software, evaluating the data from 0 to 60 min.

### 3. Results

#### 3.1. Behavioral studies

On day 15, paclitaxel-treated mice showed a significantly decreased latency to pain-related behaviors induced by a cold stimulus ( $11.5 \pm 0.9$  s) compared to control mice ( $23.4 \pm 1.7$  s) treated with vehicle (Fig. 1). The effect of single administration s.c. of three different doses of P-ITC, CP-ITC, A-ITC and NaHS on paclitaxel-treated mice is shown in Fig. 1a, b, c and d, respectively. P-ITC is active starting from  $4.43 \mu\text{mol kg}^{-1}$ . The dose of  $13.31 \mu\text{mol kg}^{-1}$  was significant starting 15 min after administration, the effect lasted until 45 min. CP-ITC ( $1.33 \mu\text{mol kg}^{-1}$ ) increased pain threshold only 15 min after administration. Otherwise,  $4.43 \mu\text{mol kg}^{-1}$  and  $13.31 \mu\text{mol kg}^{-1}$  CP-ITC increased the licking latency long lastingly. A-ITC ( $4.43$  and  $13.31 \mu\text{mol kg}^{-1}$ ) was active between 15 and 45 min after administration. NaHS was active in paclitaxel-treated mice starting from the dose of  $4.43 \mu\text{mol kg}^{-1}$ , plateauing between 30 min and 45 min after administration. The highest dose was lesser effective.

The pain threshold measurements of oxaliplatin-treated animals (Cold plate test) is shown in Fig. 2. On day 15, oxaliplatin decreased the licking latency to  $12.6 \pm 0.7$  s in comparison to control mice ( $21.5 \pm 1.2$  s). The lower dose of P-ITC induced a slight effect whereas  $4.43 \mu\text{mol kg}^{-1}$  and  $13.31 \mu\text{mol kg}^{-1}$  significantly increased the licking latency starting 15 min after administration and reaching a maximum effect 30 min after administration. CP-ITC ( $1.31 \mu\text{mol kg}^{-1}$ ) improved pain threshold between 15 and 30 min. Oxaliplatin-induced hypersensitivity was fully abolished by the high doses of CP-ITC ( $4.43 \mu\text{mol kg}^{-1}$  and  $13.31 \mu\text{mol kg}^{-1}$ ). NaHS ( $13.31 \mu\text{mol kg}^{-1}$ ) was effective between 15 and 45 min. The higher dose was lesser long-lasting. Morphine ( $21.75 \mu\text{mol kg}^{-1}$  s.c.) did not increase the licking latency of oxaliplatin-treated mice. Celecoxib ( $104.88 \mu\text{mol kg}^{-1}$  p.o.) and pregabalin ( $94.20 \mu\text{mol kg}^{-1}$  s.c.) significantly increased pain threshold only 15 min after administration. Duloxetine ( $50.43 \mu\text{mol kg}^{-1}$  p.o.) induced a maximum effect 30 min after administration (Fig. 2). Effects of these reference compounds on paclitaxel-induced neuropathic pain are already published. In particular, pregabalin and duloxetine showed similar efficacy than in oxaliplatin model (Ito et al., 2012); on the contrary, morphine (Xu et al., 2011) was partially active against paclitaxel (differently from oxaliplatin) whereas celecoxib did not (Ito et al., 2012).

P-ITC, CP-ITC and NaHS showed similar efficacy against paclitaxel- and oxaliplatin-dependent hypersensitivity when evoked by mechanical stimulus (Von Frey test, Supplementary Figs. S1 and S2).

Fig. 3a shows the comparison between the responses to acute administration s.c. of A-ITC and A-IC on oxaliplatin-treated mice. A-ITC ( $13.31 \mu\text{mol kg}^{-1}$ ) increased licking latency up to control level. On the contrary, A-IC, the analogue molecule without the sulfhydryl group (Scheme 1), was not active after administration s.c. of the same dosage. In Fig. 3b, the enhancement of pain threshold induced by a single administration of NaHS ( $13.31 \mu\text{mol kg}^{-1}$ ), P-ITC ( $4.43 \mu\text{mol kg}^{-1}$ ), CP-ITC ( $4.43 \mu\text{mol kg}^{-1}$ ), A-ITC ( $4.43 \mu\text{mol kg}^{-1}$ ) expressed as AUC (0–60 min) is shown in comparison to oxaliplatin-treated mice. The  $\text{H}_2\text{S}$ -binding molecule Hb ( $3.1 \mu\text{mol kg}^{-1}$  s.c.), co-administered with the  $\text{H}_2\text{S}$  donors, was able to fully

prevent the anti-hypersensitive efficacy. Hb *per se* was ineffective (Fig. 3b). Similar results were obtained by the non-isothiocyanate  $\text{H}_2\text{S}$  donors GYY4137 ( $4.43 \mu\text{mol kg}^{-1}$  s.c.; GYY) (Fig. 3b). In the Supplementary Fig. S3, effects over time of this morpholine-derivative were shown in the absence and in the presence of Hb.

Pain relieving activity of NaHS ( $13.31 \mu\text{mol kg}^{-1}$  s.c.), P-ITC, CP-ITC and A-ITC (all at  $4.43 \mu\text{mol kg}^{-1}$  s.c.) was fully prevented by the administration i.p. of the Kv7 potassium channel blocker XE991 ( $2.66 \mu\text{mol kg}^{-1}$ ) as shown in Fig. 4a (AUC values; 0–60 min). The relevance of Kv7 in pain modulation was confirmed through the effectiveness showed by the Kv7 opener retigabine ( $65.93 \mu\text{mol kg}^{-1}$  p.o.) in oxaliplatin-treated mice (Fig. 4b). XE991 reverted retigabine efficacy without altering the pain threshold *per se* (Fig. 4b). A dose-response curve of XE-991 *per se* effects on oxaliplatin-induced hypersensitivity is shown in the Supplementary Fig. S4.

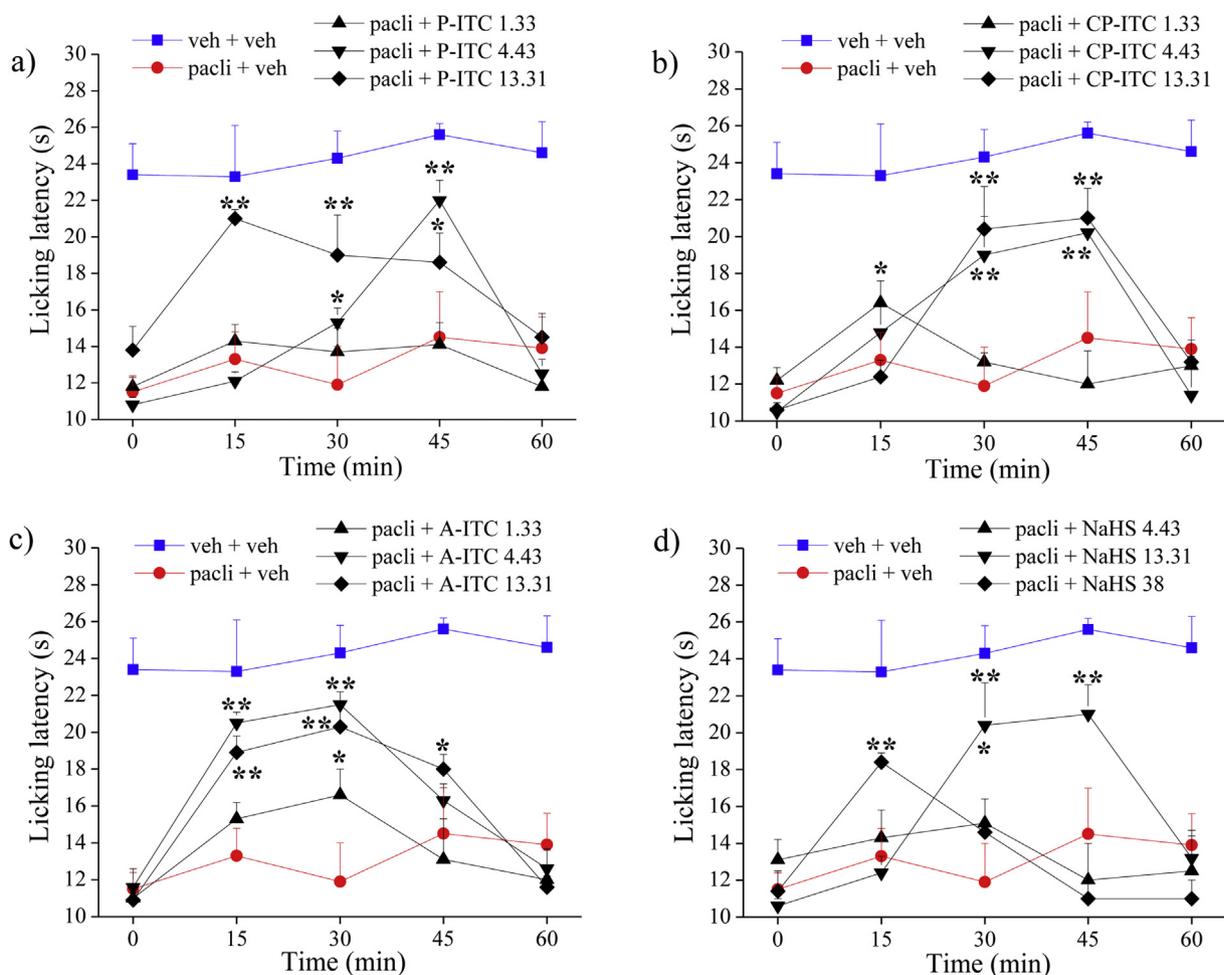
To evaluate the central component of the anti-neuropathic effect of  $\text{H}_2\text{S}$  donor molecules, the efficacy of NaHS ( $13.31 \text{ nmol/mouse}$ ) and P-ITC ( $4.43 \text{ nmol/mouse}$ ) was evaluated after i.c.v. administration in oxaliplatin-treated mice (Fig. 4c). Both compounds increased the pain threshold up to control group levels peaking 30 min after administration. These effects were fully prevented by the pre-administration of XE991 i.p. (Fig. 4c).

Neither NaHS ( $13.31 \mu\text{mol kg}^{-1}$  s.c.) nor A-ITC and P-ITC (both  $4.43 \mu\text{mol kg}^{-1}$  s.c.) were able to influence the normal pain threshold of naïve animals as evaluated by Hot plate test using a thermal noxious stimulus able to highlight analgesic effects (Supplementary Fig. S5).

#### 3.2. Electrophysiological studies

Given the *in vivo* data showing that the Kv7 channel blocker XE991 antagonized the pain-relieving activity of  $\text{H}_2\text{S}$  donors, the ability of these drugs to directly influence the activity of Kv7 channels involved in pain information processing was also investigated. To this aim, patch-clamp experiments in CHO cells transfected with Kv7.2 and Kv7.3 cDNAs were carried out. In response to voltage ramps from  $-80$  mV to  $0$  mV, heteromeric channels formed by Kv7.2/3 subunits elicit robust voltage-dependent, outwardly rectifying  $\text{K}^+$  currents. Perfusion for 1–2 min with the Kv7 opener retigabine ( $0.01$  mM) led to an increase in maximal current of  $\sim 2$ -fold, and to a leftward shift in the threshold potential of current activation of  $\sim -20$  mV (Fig. 5). Using an identical experimental paradigm, perfusion with NaHS ( $0.01$ – $10$  mM), A-ITC ( $0.01$ – $10$  mM), P-ITC ( $1$ – $100$  mM), or CP-ITC ( $0.01$ – $10$  mM) dose-dependently increased maximal current size at  $0$  mV (Fig. 5a and b) and shifted the  $\Delta V_{40}$  toward more negative membrane potential values (Fig. 5a and c); notably, the effect of CP-ITC on  $\Delta V_{40}$ , although smaller than that observed with retigabine, was significantly greater than that of NaHS. By contrast, no effect was observed upon perfusion with  $1$ – $10$  mM A-IC, suggesting that the presence of a thiol group was essential for the functional effects observed on Kv7 currents by A-ITC (Scheme 1). Finally, CP-ITC appeared more efficacious and potent than P-ITC in potentiating Kv7.2/3 maximal currents and negatively-shifting the  $\Delta V_{40}$ ; this latter result is consistent with the higher  $\text{H}_2\text{S}$  levels produced by CP-ITC when compared to P-ITC, thus highlighting a relevant role for the carboxyl group in this process (Martelli et al., 2014).

$\text{K}^+$  currents carried by Kv7.2/3 channels displayed activation kinetics which could be fitted by a sum of two exponential functions (Wang et al., 1998; Castaldo et al., 2002), with a fast and a slow time constants ( $\tau_f$  and  $\tau_s$ , respectively) (Supplementary Fig. S6, panels a and b); at any given potential above  $-40$  mV, the relative amplitude of the fast component, called  $A_f$ , largely dominated over that of the slow component, called  $A_s$ ; thus, the ratio of  $A_f$  and  $A_s$ ,



**Fig. 1.** Effects of H<sub>2</sub>S donors on paclitaxel-induced neuropathic pain. The response to a thermal stimulus was evaluated by the Cold plate test measuring the latency (s) to pain-related behaviors (lifting or licking of the paw). Mice were treated with paclitaxel (2.0 mg kg<sup>-1</sup> i.p.) on four alternate days (days 1, 3, 5 and 7) and the acute tests were performed on day 15. a) P-ITC, b) CP-ITC, and c) AITC (1.33, 4.43 and 13.31 μmol kg<sup>-1</sup>) NaHS (4.43, 13.31 and 38 μmol kg<sup>-1</sup>) were administered s.c. Measurements were performed 15, 30, 45 and 60 min after injection. Control mice were treated with vehicle. Each value represents the mean of 16 mice per group, performed in 2 different experimental sets. \*P < 0.05 and \*\*P < 0.01 vs paclitaxel + vehicle treated animals.

expressed as  $A_f/(A_f + A_s)$ , was around 1 at all potentials investigated (Supplementary Fig. S6, panel c). Perfusion with 1 mM CP-ITC failed to modify current activation rates, as no change was detected in either  $\tau_f$  or  $\tau_s$  before and after drug exposure at any given potential between  $-40$  mV and  $+40$  mV. On the other hand, the H<sub>2</sub>S donor significantly decreased the ratio between the two components (described by  $A_f/A_f + A_s$ ) for potentials  $\geq -20$  mV; this was due to a drug-dependent increase in the absolute amplitude of the slow component, virtually absent in control conditions. Perfusion with 1 mM CP-ITC also markedly reduced current deactivation kinetics (Supplementary Fig. S6, panels d and e).

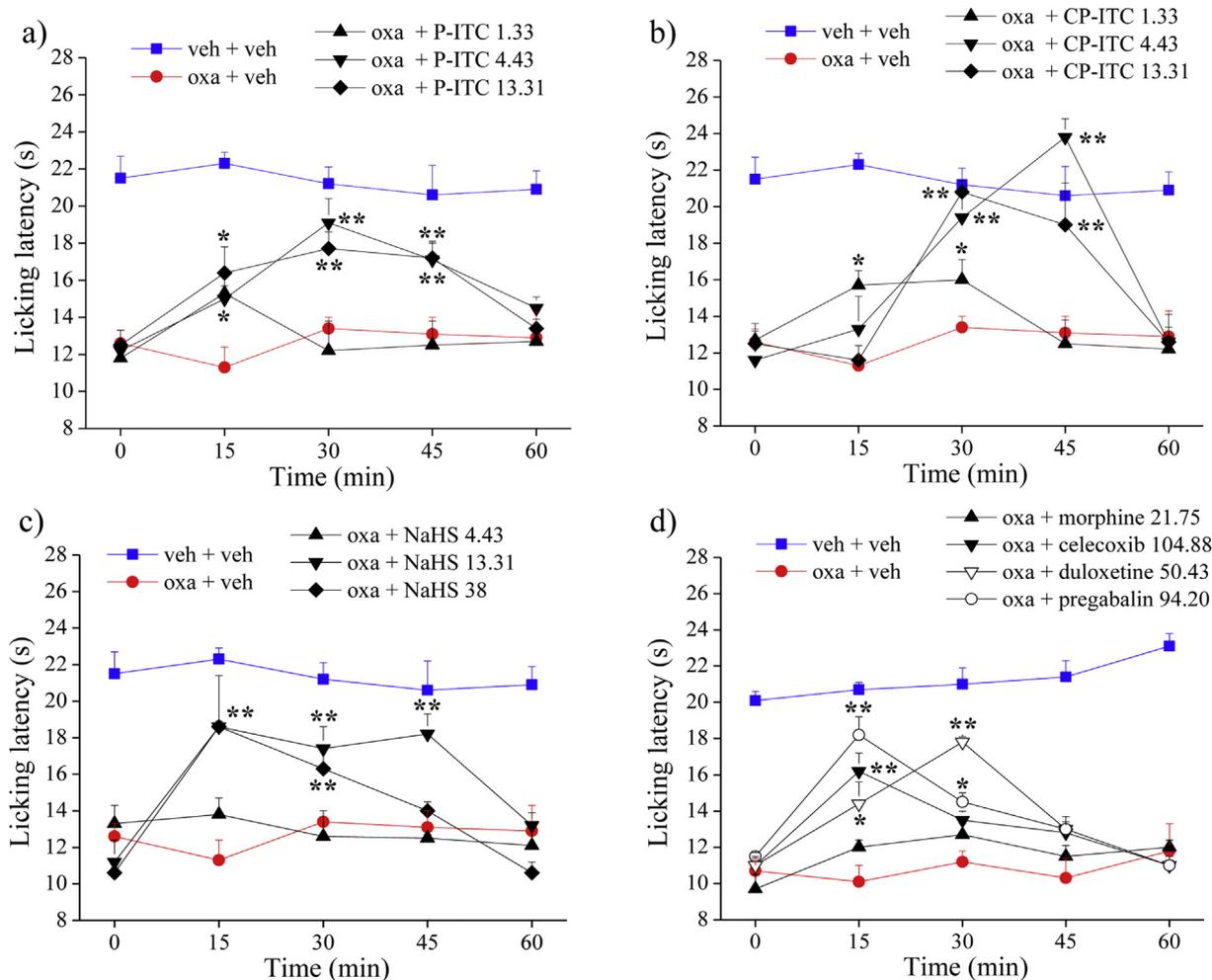
In rat hippocampal presynaptic terminals (Martire et al., 2004) and in large fibers of the rat sciatic nerve (Schwarz et al., 2006), functional Kv7.2 homomers have also been described; when compared to heteromeric Kv7.2/3 channels, Kv7.2 currents are 4–6 times smaller, activate at slightly more positive membrane voltages (Wang et al., 1998), and display a higher sensitivity to the pore blocker TEA (Hadley et al., 2000). To investigate whether H<sub>2</sub>S donors differentially affected Kv7.2 homomers when compared to Kv7.2/3 heteromers, electrophysiological experiments were also carried out in CHO cells transfected only with Kv7.2 cDNA. As shown in Supplementary Fig. S7, Kv7.2 currents showed a

pharmacological profile very similar to that of Kv7.2/3 currents, being potentiated (though with a lower potency and efficacy when compared to retigabine) by 1 mM NaHS, A-ITC, P-ITC, or CP-ITC, and being unaffected by 1 mM A-IC.

#### 4. Discussion

The development of painful neuropathies is a severe dose-limiting side effect of commonly used chemotherapeutic agents including taxanes, vinca alkaloids and platinum agents (Ward et al., 2014; Fariello et al., 2014). Paclitaxel-induced neurotoxicity is characterized by numbness, tingling and burning pain (Dougherty et al., 2004); repeated oxaliplatin administrations induce a chronic neurological syndrome that persists between and after treatment (Beijers et al., 2014; Souglakos et al., 2002) negatively influencing patient's quality of life. The pharmacological management of chemotherapy-induced neuropathy remains largely ineffective (Hershman et al., 2014).

The present results show the pain relieving efficacy of novel isothiocyanate-based H<sub>2</sub>S-donors, along with the well-known reference compounds NaHS and GYY4137, in animal models of neuropathic pain induced by anticancer drugs. P-ITC, CP-ITC and A-

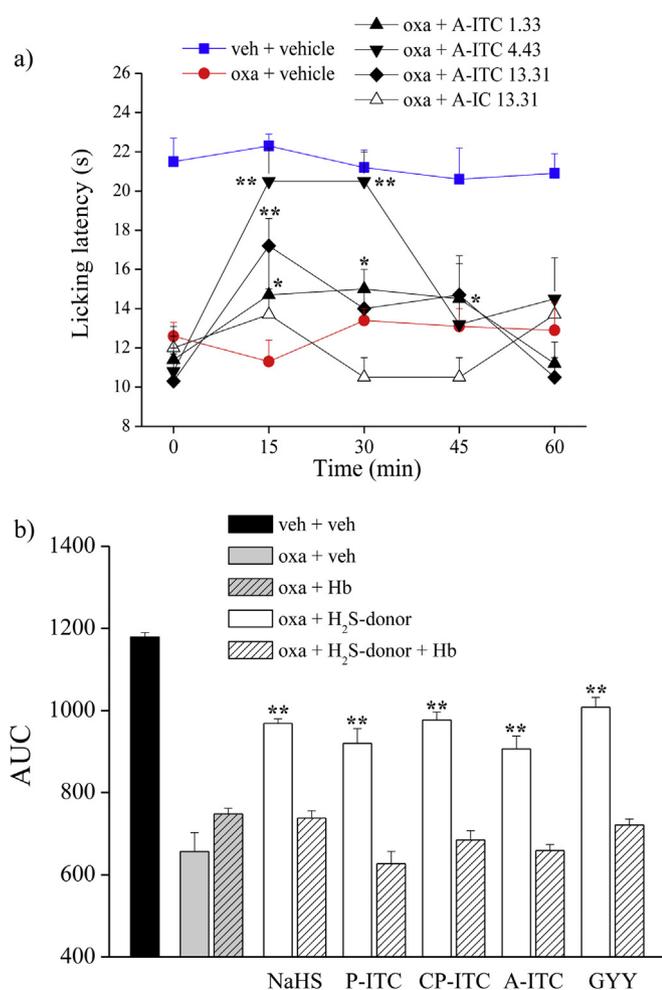


**Fig. 2.** Effects of H<sub>2</sub>S donors on oxaliplatin-induced neuropathic pain. The response to a thermal stimulus was evaluated by the Cold plate test measuring the latency (s) to pain-related behaviors (lifting or licking of the paw). Mice were daily treated i.p. with oxaliplatin 2.4 mg kg<sup>-1</sup>. Tests were performed on day 15. a) P-ITC, b) CP-ITC (both at 1.33, 4.43 and 13.31 μmol kg<sup>-1</sup>), and c) NaHS (4.43, 13.31 and 38 μmol kg<sup>-1</sup>) were administered s.c. Measurements were performed 15, 30, 45 and 60 min after injection. The reference compounds d) morphine (21.75 μmol kg<sup>-1</sup> s.c.), celecoxib (104.88 μmol kg<sup>-1</sup> s.c.), duloxetine (50.43 μmol kg<sup>-1</sup> p.o.) and pregabalin (94.20 μmol kg<sup>-1</sup> p.o.) were administered, pain threshold was assessed 15, 30, 45 and 60 min after injection. Control mice were treated with vehicle. Each value represents the mean of 16 mice per group, performed in 2 different experimental sets. \*P < 0.05 and \*\*P < 0.01 vs oxaliplatin + vehicle treated mice.

ITC reduce the hypersensitivity to a cold non-noxious stimulus (allodynia-related measurement) after the acute systemic administration of very low doses both in paclitaxel and oxaliplatin-treated animals (effectiveness has been demonstrated also using a mechanical non-noxious stimulus, both the responses are strictly related to the neuropathic condition; Di Cesare Mannelli et al., 2013) Because of the similar pharmacological profile shown in the two models, further experiments were carried out in the best characterized neuropathy model, namely that evoked by the platin derivative.

When compared to duloxetine and pregabalin, two molecules currently in clinical use to reduce the symptoms of chemotherapy-induced neuropathy (Hershman et al., 2014), isothiocyanates show similar efficacy at 10-times lower doses; morphine is ineffective against oxaliplatin-dependent pain. NaHS, a prototypical H<sub>2</sub>S-donor, shows similar efficacy as isothiocyanates, despite being less potent. Both synthetic (Martelli et al., 2014) and naturally-occurring (Citi et al., 2014) isothiocyanates act as H<sub>2</sub>S-releasing compounds, and our results are consistent with an involvement of this gasotransmitter in the antinociceptive effects shown by these molecules. The H<sub>2</sub>S-binding molecule Hb (Pietri et al., 2011) fully

prevented the anti-neuropathic efficacy of P-ITC, CP-ITC, A-ITC and NaHS, as well as of GYY4137, a morpholin-derivative able to slowly release H<sub>2</sub>S both *in vitro* and *in vivo* (Li et al., 2008). Moreover, A-IC, the corresponding isocyanate of A-ITC, unable to release H<sub>2</sub>S, was completely ineffective against oxaliplatin-induced neuropathy. Efficacy of P-ITC and NaHS was also measurable after i.c.v. administration, suggesting a central component of the H<sub>2</sub>S donors-mediated anti-neuropathic property. Independently by the route of administration, these compounds were effective at very low doses. Characteristically, while some compounds like P-ITC and CP-ITC seem to reach a maximal effect beyond which the dose increase is not significant, the anti-neuropathic effects of the highest doses of NaHS and A-ITC were less evident when compared to lower doses. Although this bell-shaped dose-dependence possibly involves the complex pharmacokinetic profile of specific H<sub>2</sub>S donors, it should be highlighted that a similar phenomenon has been also reported for the mitochondrial effects of H<sub>2</sub>S. In fact, low concentrations of H<sub>2</sub>S stimulate the mitochondrial electron transport, evoke protective effects maintaining mitochondrial function, protecting mitochondrial DNA integrity, and inhibiting mitochondrial protein oxidation (Ahmad et al., 2016). On the



**Fig. 3. Role of H<sub>2</sub>S in the relief of oxaliplatin-induced neuropathic pain.** The latency (s) to pain-related behaviors (lifting or licking of the paw) was measured by the Cold plate test. Mice were daily treated i.p. with oxaliplatin (2.4 mg kg<sup>-1</sup>) and behavior was measured on day 15. a) A-ITC (1.33, 4.43 and 13.31 μmol kg<sup>-1</sup> s.c.) was compared with A-IC (13.31 μmol kg<sup>-1</sup> s.c.). b) NaHS (13.31 μmol kg<sup>-1</sup> s.c.), P-ITC, CP-ITC, A-ITC and the morpholine derivative GYY4137 (GYG) (all at 4.43 μmol kg<sup>-1</sup> s.c.) were administered alone or in mixture with Hb (3.10 μmol kg<sup>-1</sup>). The histogram shows the average of resulting AUC. Control mice were treated with vehicle. Each value represents the mean of 16 mice per group, performed in 2 different experimental sets. \*P < 0.05 and \*\*P < 0.01 vs oxaliplatin + vehicle treated mice.

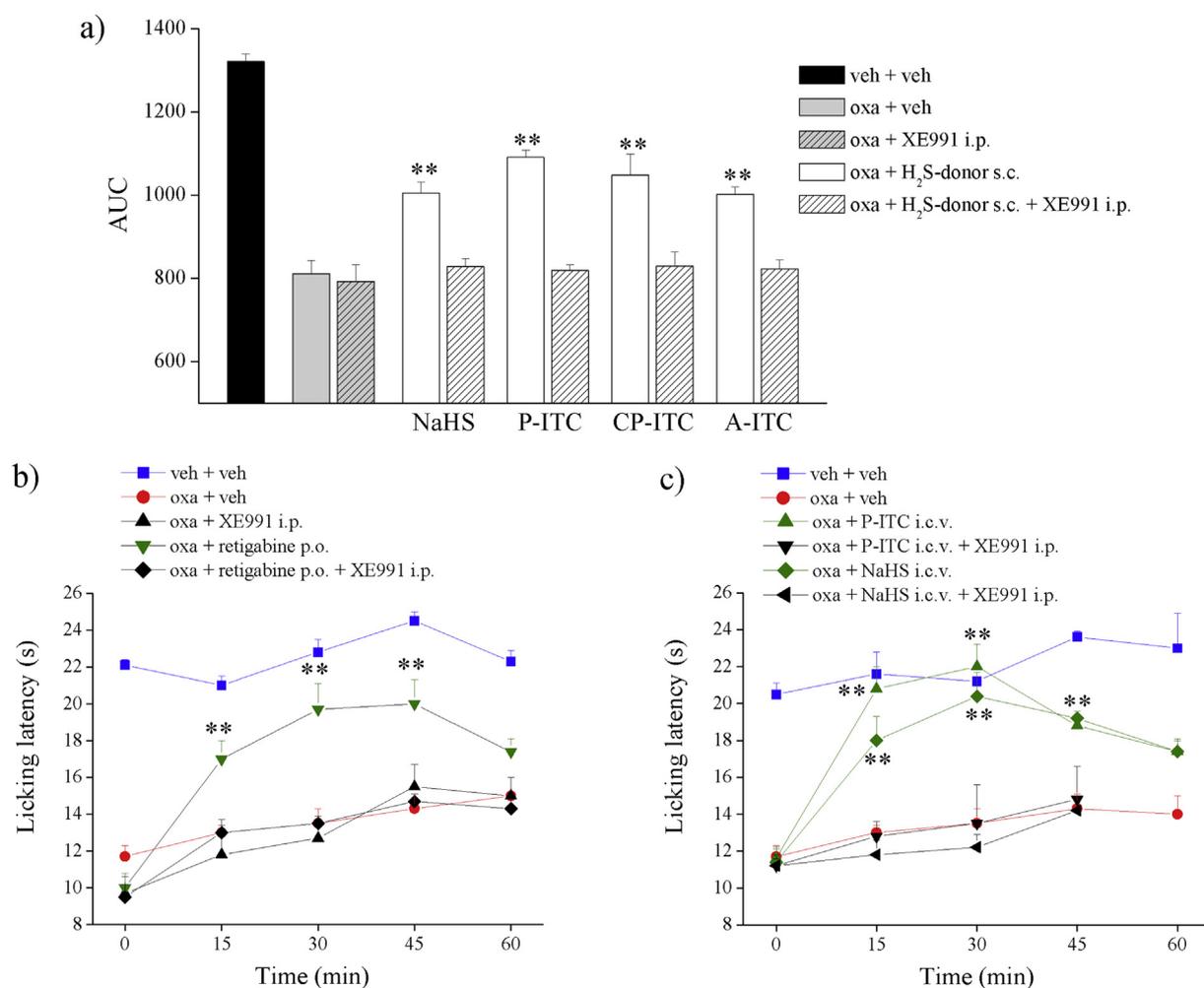
contrary, elevation of H<sub>2</sub>S concentrations beyond a certain concentration range becomes cytotoxic, pro-oxidant, and suppresses mitochondrial electron transport (Szabò et al., 2014).

The lipid-soluble nature of H<sub>2</sub>S enables this gasotransmitter to easily reach its molecular targets on the plasma membrane, inside the cytosol or in intracellular organelles. Its characteristic membrane permeability, together with its unique chemical reactivity with certain types of macromolecules in different types of cells, makes H<sub>2</sub>S a pleiotropic and powerful signaling molecule (Wallace and Wang, 2015). Voltage-gated potassium channel subunits encoded by the Kv7 subfamily have been shown to mediate a large part of the H<sub>2</sub>S-induced cardiovascular effects through an effect on Kv7.4 subunits (Schleifenbaum et al., 2010; Martelli et al., 2013a). The Kv7 gene family includes five members (Kv7.1–5), each showing a specific tissue distribution and functional role (Soldovieri et al., 2011). In particular, heteromeric assembly of Kv7.2 and Kv7.3 subunits is believed to provide a major contribution to a sub-threshold slowly-activating and deactivating, and non-

inactivating K<sup>+</sup> current known as the M-current (Wang et al., 1998), which limits repetitive firing and causes spike-frequency adaptation (Soldovieri et al., 2011). Heteromeric Kv7.2/3 channels appear as the main players in pain information processing, being abundantly expressed in nodes of Ranvier and the cell bodies of large sensory neurons as well as in the dorsal horns, the thalamus and the cortex (Wang and Li, 2016). Notably, although Kv7.5 subunits are also abundantly expressed in C-fibers and control excitability of nociceptive neurons (King and Scherer, 2012), the pharmacological profile of the M currents recorded from nociceptive DRG neurons speaks against a strong contribution of Kv7.5 (Du and Gamper, 2013). Inhibition of Kv7 channels with XE-991, a specific and potent blocker of the currents carried by Kv7 channels (Wang et al., 1998), leads to hyperexcitability of primary sensory neurons (Zheng et al., 2013). On the other hand, retigabine, an activator of neuronal Kv7 channels (Miceli et al., 2008), was neuroprotective against cisplatin- (Nodera et al., 2011) and oxaliplatin- (Abd-Elseyed et al., 2015) induced neuropathy, decreased osteo-articular and neuropathic pain (Li et al., 2008; Brown and Passmore, 2009), and suppressed the excitability of nociceptive cold-sensing trigeminal ganglion neurons (Abd-Elseyed et al., 2015). Moreover, flupirtine, a retigabine-analogue also acting as a Kv7 activator (Martire et al., 2004) suppressed axonal hyperexcitability of peripheral nerve exposed to oxaliplatin (Sittl et al., 2010). All these evidence point toward a crucial role of Kv7 channels in several models of persistent pain. In the present study, retigabine is shown to effectively protect against oxaliplatin-dependent pain, and the Kv7 blocker XE991 fully counteracted the pain relieving effects of retigabine as well as of isothiocyanates and NaHS administered by both systemic or i.c.v. routes. Importantly, XE991 reverted H<sub>2</sub>S donors effects at dosages that did not modify oxaliplatin-hypersensitivity *per se* (present data). Furthermore, XE991 did not show anti-nociceptive properties since it did not modify the normal pain threshold of naïve animals (Blackburn-Munro and Jensen, 2003; Hayashi et al., 2014). These data led us to hypothesize that H<sub>2</sub>S, by activating Kv7 channels, reduces neuronal hyperexcitability both in DRG and in central neurons, thus normalizing the altered electrophysiological activity occurring during chemotherapy-induced neuropathic pain (Di Cesare Mannelli et al., 2015b).

Patch-clamp experiments in CHO cells heterologously-expressing Kv7.2 and Kv7.3 cDNAs revealed that Kv7.2/3 heteromeric currents showed concentration-dependent activation by H<sub>2</sub>S-releasing isothiocyanates, suggesting that these channels mediate at least some of the anti-hypersensitive effects exerted *in vivo* by these compounds. Biophysical analysis of the changes introduced by the most effective H<sub>2</sub>S donor herein investigated (CP-ITC) on Kv7.2/3 activation and deactivation kinetics are suggestive of a drug-induced increased stabilization of the open configuration of the channel, leading to a decreased rate of channel deactivation, and a facilitation of channel opening by voltage. The present experiments do not allow to discriminate whether H<sub>2</sub>S activated Kv7.2/3 currents by interacting directly with the channel subunits or whether intermediate messengers are involved. It would be interesting to verify a possible interaction of H<sub>2</sub>S with radical oxygen species (ROS), given the fact that H<sub>2</sub>O<sub>2</sub> markedly increase currents formed by Kv7.2, Kv7.3, and Kv7.4 channels (Gamper et al., 2006). In Kv7.4 channels, a conserved cysteine triplet in the S2–S3 linker was identified as critical for this pharmacological effect; whether this same site is also involved in the described effects of H<sub>2</sub>S on Kv7.2/3 channels is still unknown.

It should be noted that all the isothiocyanates used in this study, along with the reference H<sub>2</sub>S donor NaHS, showed anti-neuropathic effects. However, they exhibited different degrees of efficacy and potency (in the *in vivo* experiments and in the electrophysiological studies), not clearly related with their H<sub>2</sub>S-



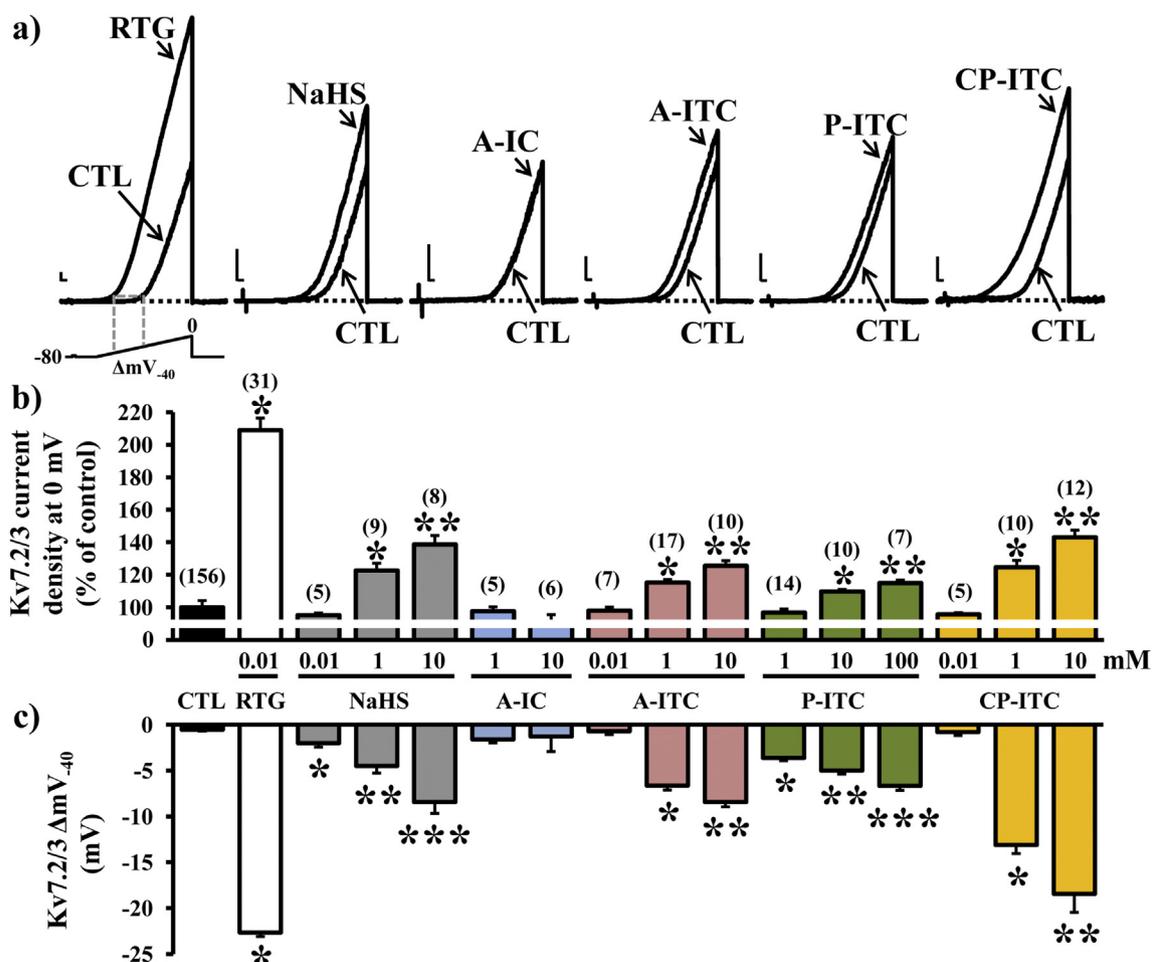
**Fig. 4. Involvement of Kv7 channels in H<sub>2</sub>S-mediated anti-neuropathic effect.** The latency (s) to pain-related behaviors (lifting or licking of the paw) was measured by the Cold plate test. Mice were daily treated i.p. with oxaliplatin (2.4 mg kg<sup>-1</sup>) and behavior was measured on day 15. a) P-ITC, CP-ITC, A-ITC (4.43 μmol kg<sup>-1</sup>) and NaHS (13.31 μmol kg<sup>-1</sup>) were administered s.c. XE991 (2.66 μmol kg<sup>-1</sup>) was administered i.p. 15 min before H<sub>2</sub>S-donors. The histograms show the average of resulting AUC. b) Retigabine (2.66 μmol kg<sup>-1</sup>) was administered p.o. in the absence and in the presence of a pretreatment (15 min before) with XE991 (2.66 μmol kg<sup>-1</sup> i.p.). c) P-ITC (4.43 nmol/mouse) and NaHS (13.31 nmol/mouse) were administered i.c.v. in the absence and in the presence of a pretreatment (15 min before) with XE991 (2.66 μmol kg<sup>-1</sup> i.p.). Control mice were treated with vehicle. Each value represents the mean of 16 mice per group, performed in 2 different experimental sets. \*P < 0.05 and \*\*P < 0.01 vs oxaliplatin + vehicle treated mice.

releasing profiles. Slow H<sub>2</sub>S-donors, such as GYY4137, are often more effective than rapid donors, such as NaHS, suggesting that long-lasting generation of relatively lower concentrations of H<sub>2</sub>S can be preferable for certain pharmacological effects (Li et al., 2008). Moreover, the isothiocyanates used in this study are not “spontaneous” H<sub>2</sub>S-donors: indeed, the H<sub>2</sub>S-release from these compounds is triggered by the presence of organic thiols such as L-cystein (Martelli et al., 2014). Thus, this process can be “smartly” promoted in biological environments by a large availability of endogenous organic thiols (and perhaps by other metabolic reactions), but can be also influenced by variables linked to different experimental models which can make difficult the quantitative correlation between H<sub>2</sub>S-release and pharmacological effects. Finally, even pharmacokinetic aspects, concerning the ability of the H<sub>2</sub>S-prodrugs to reach the pharmacological target, may play a role.

The emerging data on the biological effects of H<sub>2</sub>S, and the etiopathogenic roles of both excessive and defective H<sub>2</sub>S biosynthesis in some diseases, support two main approaches for the development of sulfide-based therapeutics: suppression of endogenous sulfide formation by inhibitors of CSE and CBS, and gaseous H<sub>2</sub>S or H<sub>2</sub>S-releasing compounds (sulfide donors). The

formers (DL-propargylglycine and β-cyanoalanine) have low potency and a questionable selectivity (Szabó, 2007). Concerning the latter compounds, excluding gaseous H<sub>2</sub>S for its poor safety, H<sub>2</sub>S equivalents or releasing agents (i.e. donors) are becoming important research tools (Zhao et al., 2014). In the past, some series of different H<sub>2</sub>S pro-drugs were developed (Zheng et al., 2015; Zhao et al., 2014) often obtaining a limited control of gas release. Presently, there is still scarce heterogeneity of H<sub>2</sub>S-releasing moieties endowed with satisfactory pharmacological and chemical (stability) features. Isothiocyanate exhibit interesting H<sub>2</sub>S-releasing properties which can be usefully employed for therapeutic purposes. The effectiveness of allyl isothiocyanate, mainly obtained from the seeds of *Brassica nigra* L., raises the intriguing possibility to exploit the plant kingdom as a source of H<sub>2</sub>S-based therapeutics against neuropathic pain.

In this perspective, the present study demonstrates that H<sub>2</sub>S-releasing compounds, such as the “gold standard” GYY4137 or the novel isothiocyanates, promote H<sub>2</sub>S-mediated activation of Kv7 channels and may represent an exciting and innovative approach to drug-resistant forms of pain such as chemotherapy-induced neuropathies.



**Fig. 5.** Effect of H<sub>2</sub>S donors on Kv7.2/3 currents heterologously-expressed in CHO cells. a) Macroscopic currents recorded in response to a voltage ramp from  $-80$  to  $0$  mV in CHO cells expressing Kv7.2/7.3 channels in control solution (CTL) or after perfusion with the maximal concentration tested of each of the following compound (in mM): 0.01 retigabine (RTG), 10 NaHS (NaHS), 10 allyl-isocyanate (A-IC), 10 allyl-isothiocyanate (A-ITC), 100 phenyl-isothiocyanate (P-ITC), or 10 3-carboxyphenyl-isothiocyanate (CP-ITC), as indicated. Current scale: 200 pA; time scale: 200 ms. b and c) Quantification of the effects prompted by each compound on peak current increase (b) or  $\Delta V_{40}$  (c). Single asterisks indicate, for each drug, values significantly ( $P < 0.05$ ) different versus controls; double and triple asterisks indicate values significantly different ( $P < 0.05$ ) versus controls and versus values recorded upon perfusion of a lower drug concentration. The numbers on top of each bar indicate the number of measurements, each from a different cell.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2017.04.029>.

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