



Naturally occurring glucosinolates and isothiocyanates as a weapon against chronic pain: potentials and limits

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Received: 1 September 2021 / Accepted: 2 February 2022 / Published online: 22 February 2022
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Abstract Investigation into glucosinolates (GLs) therapeutic effects boasts a long history, which began with the evidence that their hydrolysis-derived isothiocyanates (ITCs) could exert cytoprotective effects through the modulation of both the inflammatory response (NF- κ B pathway) and the oxidative stress (Nrf2/ARE pathway), two processes largely involved in the pathogenesis of chronic pain syndromes. GLs and ITCs are also able to modulate the activity and the expression of several targets involved in pain regulation, like opioid receptors. Recently, ITCs turned out to be slow- H_2S donors *in vivo*, able to directly modulate the activity of a subtype of K_V7 potassium channels involved in the transmission of painful stimuli, providing a further incentive to their employment in pain management. Nevertheless, some controversies exist in the use of ITCs for pain relief considering their ability to positively modulate the activity of TRPA1 receptors. This review focuses on the preclinical and clinical evidence attesting the beneficial effects of GLs and their derivatives ITCs in chronic inflammatory and neuropathic conditions. In this context, the mechanisms underlying the ability of GLs and ITCs to modulate pain perception and,

besides, to prevent the establishment of chronic pain will be described along with their pharmacokinetics and toxicological profile. Finally, other possible mechanisms hidden behind GLs efficacy on pain will be discussed.

Keywords Glucosinolates · Isothiocyanates · Pain · H_2S · Neuropathy · Inflammation

Chronic pain

“*Divinum opus est sedare dolorem*” (Hippocrates).

Pain represents a clinical burden, considering its high prevalence among patients and the lack of adequate therapies in most cases (Mäntyselkä et al. 2001). Although pain itself is not life-threatening, the development of chronic pain has a strong impact on quality of life, therefore its management represents a health priority in patients (Goldberg and McGee 2011). Chronic pain can manifest in two different forms, namely as a primary or secondary syndrome. Primary chronic pain is considered a proper disease whereas, chronic secondary pain arises as a symptom of another disease (cancer, diabetic neuropathy, inflammatory bowel disease, or arthritis), though it continues after the healing of the underlying disease (Treede et al. 2019). It has been estimated that the development of chronic pain occurs in $\sim 20\%$ of the adults in Europe (Breivik et al. 2006) as well as in US

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(Dahlhamer et al. 2018) and it is accompanied by a huge health and economic burden (Barham 2012; Gaskin and Richard 2012).

Chronic pain syndromes can differ in the aetiology, modes of manifestations, and response to pain medication (Treede et al. 2019). Chronic musculoskeletal pain, affecting bones, joint or muscles (Perrot et al. 2019) can result from inflammatory processes, autoimmune, or metabolic diseases, or can be secondary to motor nervous system diseases (e.g., Parkinson's disease) (Nicholas et al. 2019). Besides, neuropathic pain is due to a direct damage to the nervous system, like that caused by tissue injury, metabolic disorders (diabetes), infections (HIV, herpes zoster), chemotherapeutic agents (platinum drugs, taxanes, vinca alkaloids) or alcohol abuse (Jensen et al. 2011). The etiopathogenesis of neuropathic pain makes it unresponsive to almost all the painkiller drugs (Treede et al. 2008). Finally, chronic pain can also originate from the viscera (e.g., intestine or uterus) as a consequence of mechanical factors (obstruction), vascular mechanisms (ischemia), or persistent inflammation (Aziz et al. 2019; Häuser et al. 2020). Visceral pain can also be related to a disruption of the microbiota homeostasis (O'Mahony et al. 2017; Vila et al. 2018) or to the presence of cancer in internal organs (Bennett et al. 2019).

The treatment of chronic pain depends on pain subtype. Conditions primarily associated with inflammation (e.g., osteoarthritis) are preferably treated with acetaminophen and non-steroidal anti-inflammatory drugs, whereas pain with a neuropathic base is treated with tricyclic antidepressants, selective-serotonin/noradrenaline reuptake inhibitors, and Alpha 2 Delta ($\alpha 2\delta$) ligands, namely gabapentin and pregabalin (Hylands-White et al. 2017). Despite the large number of medications available for the treatment of pain, their limited efficacy in chronic conditions and several side effects, particularly in the elderly (Crofford 2013), led to investigate novel strategies to safely employ in long-term therapies. Among them, many natural products have been investigated with the aim of combining different mechanisms of action to achieve greater efficacy. The broad range of beneficial effects (anti-inflammatory, antioxidant, neuroprotective, and anti-hyperalgesic) endowed by glucosinolates (GLs), make them the ideal natural tool in the management of chronic pain syndromes, which in most cases have a complex aetiology.

Glucosinolates and isothiocyanates

Glucosinolates (*S*- β -thioglucoside *N*-hydroxysulfate; GLs) are sulfur-containing phytochemicals mainly found in cruciferous (*Brassicaceae*), most of which are edible plants (Holst and Williamson 2004a). GLs content in these vegetables depends on several factors including the cultivation region and conditions, the plant part, the degree of development, as well as genetic and environmental factors. In foliage GLs content range from 1000 to 3000 ppm (Brussels sprouts), while the concentrations of GLs in roots and seeds can be higher (30,000–60,000 ppm) (Agerbirk and Olsen 2012). Starting from sinalbin (Robiquet and Boutron 1831), hundreds of different GL structures were discovered in nature (Sønderby et al. 2010), which differ each other for the precursor as well as for the secondary modifications occurring on their sugar moieties (e.g. oxidation, methoxylation, sulfation, glucosylation) (Agerbirk and Olsen 2012; Radojčić Redovniković et al. 2008). In plants, the β -thioglucosidase enzyme myrosinases (EC 3.2.1.147) is responsible for GLs metabolism. This enzyme, which is physically segregated from GLs in plants, once released after mastication, cutting or cooking, leads to the hydrolysis of GLs into glucose and the unstable aglycones, which then turn into isothiocyanates (ITCs) or indoles, based on their side chain. In mammalian tissues conversion of GLs to ITCs is possible thanks to the gastrointestinal bacteria (Shapiro et al. 1998). Besides, several physico-chemical factors (e.g., pH, the number of double bonds in the side chains, the presence of ferrous ions, the epithiospecific protein), instead determine the conversion of ITCs and indoles into other compounds, such as ephthionitriles, nitriles and thiocyanates. Nevertheless, ITCs have been recognized as the active compounds responsible for the effects of GLs on human health, which are likely the results of a plurality of molecular mechanisms, (modulation of xenobiotic metabolism and inflammation, the regulation of cell cycle and oxidative stress, as well as the regulation of epigenetic events), many of which participate to chronic pain development and persistence (Capuano et al. 2017). Moreover, recent evidence attested the ability of GLs and ITCs to modulate the activity of channels and receptors involved in pain transmission (Lucarini et al. 2018b, 2019a). In the next paragraphs we describe in detail the beneficial effects of these phytochemicals

and their positive implications in the treatment of chronic pain.

GLs, ITCs and pain

Brassicaceae family encloses several plants which have been employed in medicine as analgesics. Among the *Brassicaceae*-derived phytochemicals, the most studied in the context of pain are glucoraphanin and its derived ITC, sulforaphane, whose effects on pain involve the activation of Nrf2, as well as the inhibition of the NF- κ B signalling pathway (as described in details in the following sections), and therefore the downregulation of cytokines expression (Guerrero-Beltrán et al. 2012b). Sulforaphane has been observed to relieve pain associated with diabetes or osteoarthritis in preclinical models, in part, by modulating the inflammatory response (Davidson et al. 2013; Negi et al. 2011). Sulforaphane has been also reported to upregulate the expression of opioid- μ receptor (Wang and Wang 2017) and enhance morphine analgesic effect (Ferreira-Chamorro et al. 2018; Redondo et al. 2017). In a similar manner, broccoli sprouts and sulforaphane were able to reduce abdominal pain by the activation of μ opioid receptors, as proven by naltrexone-mediated inhibition (Distrutti et al. 2010). The same compounds demonstrated to have further modulatory activity on visceral motor functions in vitro where they showed a spasmolytic activity on pig ileum similar to papaverine (Guadarrama-Enríquez et al. 2018). Accordingly, sulforaphane has been reported to alleviate bone pain and enhance morphine potency in rats with cancer. Interestingly, also in this case, the anti-hyperalgesic effects of sulforaphane were partially blocked by opioid receptor antagonists (Fu et al. 2021). ITC moiety might influence opioid receptors activity by irreversibly binding the sulfhydryl groups on their constituent amino acids (cysteine or lysine), which may control receptors conformation and functionality. Anyway, it has been observed that the introduction of the ITC-group into 1-position of fentanyl analogues, increased their selectivity for δ opioid receptor but decreased their analgesic activity (Bi-Yi et al. 1999).

Di Cesare Mannelli et al. (2017) and Lucarini et al. (2018a, b) observed that synthetic (allyl-, phenyl- and 3-carboxyphenyl-ITC) and natural (sulforaphane) ITC-based compounds, were able to counteract

chemotherapy-induced neuropathic pain, showing a similar efficacy and a tenfold greater potency (10-times) than duloxetine and pregabalin, the reference drug in the management of neuropathies associated with chemotherapy (Hershman et al. 2014). Noteworthy, morphine is ineffective against oxaliplatin-dependent pain (Mannelli et al. 2017), thus excluding opioid receptors from the mechanisms underlying ITCs-mediated effects. Interestingly, these compounds have been described as slow H₂S donors (Citi et al. 2014; Martelli et al. 2014). The involvement of H₂S release in the antinociceptive effects of ITCs has been proven by the fact that co-administering two H₂S scavengers, like haemoglobin and glutathione, strongly reduced their pain-relieving efficacy (Mannelli et al. 2017). At the same time, the antinociceptive effects of ITCs was almost completely reverted by XE991 (a selective blocker of Kv7 channels). Taken together this evidence suggest that the activation of Kv7 potassium channels might account for the H₂S-mediated anti-hyperalgesic effects of ITCs. Subsequently, the efficacy of ITCs against chronic pain has been confirmed in other preclinical models of neuropathy induced by ligation of sciatic nerve or diabetes, respectively, as well in the experimental model of osteoarthritis induced by injecting monoiodoacetate into joint space (Lucarini et al. 2018a, 2019b). Moringin, a natural ITC present in *M. oleifera* Lam., effectively reduced the typical neuropathic pain associated to multiple sclerosis in mice (Giacoppo et al. 2017a). Likewise, a series of semi-synthetic derivatives of *Moringa* ITCs showed a dose-dependent antinociception in a model of formalin-induced joint inflammation (Dos Santos et al. 2018).

Some natural ITCs (such as allyl-ITC, AITC) were reported to modulate the activity of TRPA1 channels which are known to be involved in the pathophysiology of several painful conditions (Logashina et al. 2019). Anyway, high micromolar concentrations of AITC are employed to reproduce animal models of pain, and itch (Andersen et al. 2017; Martelli et al. 2020a). This implies that AITC concentrations need to be increased further to induce TRPA1 desensitization and analgesia, thus excluding the possibility that TRPA1 activation is the main mechanisms of action by which ITCs can relieve pain. The activation of TRPA1 channels, as well as the opening of voltage-dependent calcium channels, also fuels the controversy about the role played by H₂S on pain regulation

(Roa-Coria et al. 2019; Tsubota-Matsunami et al. 2012), though the gasotransmitter has been reported to activate TRPA1 channels at concentrations much higher than the physiological one (Logashina et al. 2019).

In addition to exert an acute antihyperalgesic effect, the repeated treatment with glucoraphanin and sulforaphane was able to counteract the development of neuropathic pain caused by the administration of a chemotherapeutic agent in mice (Lucarini et al. 2018b). As well, sulforaphane intrathecally administered resulted able to counteract inflammation and oxidative stress caused by spinal nerve transection, concomitantly blocking the development of neuropathic pain (Kim et al. 2010). These properties can be attributed to the well-known neuroprotective effects of GLs. In another work, a defatted seed meal of *Eruca sativa*, enriched in GLs (mainly glucoerucin), resulted effective in relieving neuropathic pain caused by diabetes in mice after an acute administration, without reverting neuropathic pain after a repeated treatment (Lucarini et al. 2019b). This evidence confirms that administering GLs can counteract the pathophysiological mechanisms underlying neuropathy development, but it is much less effective in reverting neuropathy when it is already established, highlighting the importance of using these compounds from the earliest stages of the disease. Moreover, the animals developed no tolerance to the anti-hyperalgesic effect of *Eruca sativa* meal after the repeated treatment (Lucarini et al. 2019b). This represents an great advantage over treatment with others pain-relieving drugs, such as opioids (Christie 2008). In the next paragraphs we will deepen the mechanism which have been proven to be involved in the anti-hyperalgesic effect of GLs, as well as the mechanisms which might participate in it.

GLs as anti-inflammatory/antioxidants agents

Pain is a cardinal feature of inflammation and, conversely, the persistence of pain is often caused by a chronic neuroinflammation. Non-neuronal cells, such as immune cells and glial cells, actively involved in the regulation of inflammatory responses, participates in the pathogenesis and persistence of chronic pain (Ji et al. 2016). On this base, there appears to be clear

therapeutic potential for chronic pain management of compounds endowed with strong anti-inflammatory and antioxidant activities, like GLs and ITCs, which effectively counteracted inflammatory processes in different in vitro and in vivo models of disease. GLs and their metabolites, ITCs and indoles, have been shown to exert multiple chemopreventive effects (Table 1). Indeed, ITCs and indoles regulate the activity of both Nuclear erythroid 2-related factor 2 (Nrf2) and Nuclear factor- κ B (NF- κ B) which have a main role in oxidative stress and inflammation, respectively (Esteve 2020; Guerrero-Beltrán et al. 2012a; Wu et al. 2004). These phytochemicals can inhibit nuclear factor NF- κ B translocation, preventing proinflammatory cytokine production (IL-1 β and TNF- α) and andoxidative species generation (i-NOS, nitrotyrosine and PARP) (Saleh et al. 2021). Oxidative stress and inflammation are tightly connected, one of which can be triggered by the other (Biswas 2016), they thus occur together in a large number of diseases. Moreover, redox imbalance contributes to consolidate pain by exacerbating inflammatory responses (Gunn et al. 2020; Kaushik et al. 2020; Marchev et al. 2017; Sánchez-Domínguez et al. 2015). Anyway, increasing evidence in literature attests that oxidative stress has a key role in the pathogenesis of neuropathic pain in which inflammatory processes are not necessarily involved (Areti et al. 2014; Hosseini and Abdollahi 2013; Mannelli et al. 2012; Naik et al. 2006). ITCs can either directly interact with sulfhydryl residues on Keap1, the cytoplasmatic repressor of Nrf2, determining the release of the factor and its translocation into the nucleus, or activate the MAPK pathway, indirectly causing the phosphorylation of Keap1 and release of Nrf2 (Hu et al. 2004). Once translocated into the nucleus, Nrf2 activates ARE-responsive genes and induces the phase II response (Dinkova-Kostova et al. 2002). However, the sole anti-inflammatory or antioxidant activity displayed by these phytochemicals can not entirely explain their pain-relieving efficacy, and this led to the search for additional mechanisms.

GLs as neuroprotective agents

Oxidative stress and chronic inflammation are commonly partners in the pathogenesis of central nervous system diseases, against which both GLs and ITCs exerted protective effects (Table 2). Among GLs and

Table 1 Antioxidants and anti-inflammatory effects of the sources of GLs and ITCs

Source of GLs	Effects	Subjects	References
<i>Raphanus sativus</i> (Radish) extracts from the aerial and underground parts	Disease recovery or improvement	Folk medicine-based management of stomach disorders, urinary infections, hepatic inflammation, cardiac disorders, and ulcers	Manivannan et al. (2019)
<i>Moringa oleifera</i> (Moringa) water extract from leaves	Attenuated expression of iNOS and IL-1 β and production of nitric oxide and TNF α	Macrophages	Waterman et al. (2014)
<i>Armoracia rusticana</i> (Horseradish) extracts from roots	Anti-inflammatory properties	LPS-stimulated macrophages	Marzocco et al. (2015)
<i>Nasturtium officinale</i> R. Br (Watercress) extract containing standardized GLs	Decrease of lipid peroxidation, protein carbonyl, catalase, superoxide dismutase, and C-reactive protein levels	People with physical disabilities	Clemente et al. (2020)
<i>Matthiola arabica</i> ITCs rich fraction	Reduction of oxidative stress, inflammatory and fibrosis markers	Rat model of liver fibrosis	Mohammed et al. (2017)
Magliasa traditional Iranian formula from seeds of <i>Lepidium sativum</i> , <i>Linum usitatissimum</i> , and <i>Allium ampeloprasum</i> cv. Porrum, the fruit of <i>Bunium persicum</i> and <i>Terminalia chebula</i> , and the gum resin of <i>Pistacia lentiscus</i>	Improvement of the colonic histopathological score with a reduction of TNF- α , IL-1 β , MPO and lipid peroxidation in the gut	Rat model of colitis	Rahimi et al. (2013)
Lactic acid bacteria broth enriched with <i>Eruca sativa</i> (Rocket) extract from seeds	Pathogen-induced intestinal inflammation (CXCL8) and barrier dysfunction	Caco-2 cells infected with <i>Escherichia coli</i>	Bonvicini et al. (2020)
GLs-rich diet	Up-regulation of the expression of typical Nrf2 target genes like Nqo1, Gstm1, Srxn1, and GPx2 in the colon	Mice with inflammatory damage and tumour genesis	Lippmann et al. (2014)
3,3'-Diindolylmethane, GL naturally occurring in <i>Brassicaceae</i>	Anti-inflammatory properties	Adipocytes co-cultured with macrophages	Lopez-Vazquez et al. (2017)
Phenethyl ITC, hydrolysis product of gluconasturtiin	Anti-inflammatory properties	Psoriasis-like skin lesions in mice	Lee et al. (2011)
Allicin	Improvement of the colonic histopathological score with a reduction of IL-1b and TNF-a	Rats with colitis	Li et al. (2015)
Sinigrin	Block of the activation of p-38 and JNK pathway Suppression of NF- κ B/MAPK pathways Block of NLRP3 inflammasome activation	Caco-2 cells stimulated by IL-1b Macrophages	Lee et al. (2017)
Allyl nitrile	Upregulation of antioxidant/phase II enzymes in various tissues		Tanii (2017a, b)
Sulforaphane-rich broccoli sprouts	Nrf2-dependent antioxidant and anti-inflammatory protection	C57BL/6 female mice infected with <i>H. pylori</i>	Yanaka et al. (2009)

Table 1 continued

Source of GLs	Effects	Subjects	References
Sulforaphane-rich broccoli sprouts juice	Increased trans-epithelial electrical resistance, a parameter reflecting the functionality of the tight junctions and the integrity of the cell monolayer	In vitro model of inflamed human intestinal epithelium	Ferruzza et al. (2016)

their hydrolytic products, glucoraphanin, sulforaphane, moringin, phenethyl ITC, 6-(methylsulfinyl) hexyl ITC, and erucin (Jaafaru et al. 2018) showed interesting properties as modulators of oxidative stress, inflammation, and apoptosis, resulting in neuroprotective effects in different animal models (Table 2).

The most studied ITCs in neurodegenerative diseases are sulforaphane and moringin (4-(α -l-rhamnosyloxy)-benzyl ITC), derived from the hydrolysis of the GLs glucoraphanin and glucomoringin, which showed neuroprotective activity in preclinical models of neurodegeneration due to their capacity to modulate neuronal functions (Dinkova-Kostova and Kostov 2012; Tarozzi et al. 2013).

The neuroprotective effects mediated by ITCs have been mainly attributed to Nrf2/ARE pathway positive modulation (Mein et al. 2012). Nevertheless, several recent studies reported multiple other mechanisms, including inhibition of cytochrome P450 enzymes, induction of apoptosis and cell cycle arrest, and anti-inflammatory effects. These mechanisms have been proposed to work synergistically to provide the observed neuroprotective effects of ITCs (Giacoppo et al. 2015b).

In several studies carried out in vivo and in vitro on models of nervous system diseases, the treatment with GLs and ITCs have been reported to modulate the activity not only of neurons but also of glial cells, mainly astrocytes and microglia (Galuppo et al. 2013; Latronico et al. 2021; Venditti and Bianco 2020). Glial cells are resident in the periphery as well as in the central nervous system accompanying the path of pain signaling. Noteworthy, it has been demonstrated that these cells play a key role in chronic pain pathophysiology, irrespective to their location (Ji et al. 2013; Lucarini et al. 2020, 2021b; Milligan and Watkins 2009). In the periphery, also enteric glia is emerging as

a key regulator of visceral sensitivity (Lucarini et al. 2021b; Morales-Soto and Gulbransen 2019). Although the effect of GLs and ITCs on enteric nervous system has not been investigated yet, the dietary intake of these phytochemicals might improve gastrointestinal sensory functions, like pain, by modulating glia activation. Indeed, we cannot exclude that GLs and ITCs might act via a yet undiscovered pathway to influence pain signalling and protect neuronal functions.

GLs and H₂S-releasing properties

In the last years great importance has been attributed to the hydrogen sulfide (H₂S) releasing capacity of GLs-derived natural and synthetic ITCs (Lucarini et al. 2018b; Mannelli et al. 2017; Martelli et al. 2020b). As a gas transmitter, H₂S, freely crosses cell membranes and interact with different cellular and molecular targets by three main mechanisms: interaction with metal center scavenging of reactive oxygen species and reactive nitrogen species, or proteins S-persulfidation (Filipovic 2015; Ono et al. 2014; Pietri et al. 2011; Spassov et al. 2017).

H₂S is endowed of important physiological functions in the cardiovascular system as well as in nervous system, which fuelled the scientific community interest in the possibility to exploit H₂S for therapeutic purposes (Xiao et al. 2018). Nevertheless, H₂S is difficult to directly handle because it is a toxic and flammable gas. This problem pushed the research to the identification of moieties that would allow to control the dose, duration, timing, and site of H₂S release (Martelli et al. 2020b). Among them, the ITC moiety is one of the most promising. Notably, a close overlap between numerous biological effects (antioxidant, anti-inflammatory, potassium channels activity

Table 2 Neuroprotective effects of the sources of GLs and ITCs

Source of GLs	Effects	Subjects	References
RS-glucoraphanin bioactivated with myrosinase	Decreased nuclear factor (NF)- κ B translocation, pro-inflammatory cytokine (IL-1 β) production, and apoptosis (Bax and caspase 3 expression)	Mouse model of multiple sclerosis (EAE)	Giacoppo et al. (2013)
RS-glucoraphanin bioactivated with myrosinase	Reduced inducible nitric oxide synthase expression (iNOS), intercellular adhesion molecule 1 (ICAM-1), nuclear factor (NF)- κ B translocation and apoptotic pathway triggering	Rat model of cerebral ischemia/reperfusion injury	Giacoppo et al. (2014b)
RS-glucoraphanin bioactivated with myrosinase	Decreased histological damage Decreased astrocytes (GFAP-positive cells) activation	Mouse model of spinal cord injury	Galuppo et al. (2013)
RS- glucoraphanin	Counteraction of Blood Brain Barrier alteration by preserving tight junctions integrity	Mouse model of multiple sclerosis (EAE)	Giacoppo et al. (2014a)
Sulforaphane and glucoraphanin containing- food	Prevention of depression-like phenotype development	Rat undergoing repeated social defeat stress	Yao et al. (2016)
2-Phenethyl ITC and sulforaphane	Reduced ROS production induced by H ₂ O ₂ Inhibition of MMP-2 and MMP-9 expression and activity induced by LPS. Inhibition of ERK activity	Primary cultures of rat astrocytes	Latronico et al. (2021)
Sulforaphane	Reduced ROS formation	SH-SY5Y cells treated with 6-OHDA	Tarozzi et al. (2013)
Sulforaphane	Activation of ERK1/2 and Nrf-2 pathway	5-S-cysteinyldopamine-induced toxicity in cortical neurons	Vauzour et al. (2010)
Sulforaphane	Improvement of motor nerve conduction velocity, nerve blood flow, and pain Increased expression of Nrf2 Lowered NF- κ B expression and IKK phosphorylation Abrogation of (iNOS) and COX-2 expression. Reduced TNF- α and IL-6 levels	Neuro2a cells and sciatic nerve of diabetic animals	Negi et al. (2011)
Sulforaphane	Enhanced aquaporin-4 expression and decreases cerebral oedema	Rodent model of traumatic brain injury	Dash et al. (2009, Zhao et al. (2005a)
Sulforaphane	Protection of retinal function	Retinal ischemic injury	Ambrecht et al. (2015)
Sulforaphane	Reduced number of apoptotic cells in the retina epithelial cell layer, enlarged a- and b-wave amplitudes and delayed photoreceptor degeneration	Light-induced damage of the retina	Kong et al. (2007), Tanito et al. (2005)
Sulforaphane	Augmented levels of heme oxygenase 1 and reduced density of microglial cells in the hippocampus Attenuated production of iNOS, IL-6, and TNF- α	Mice model of neuroinflammation induced by LPS	Innamorato et al. (2008)
Sulforaphane	Nrf2-dependent induction of the phase II antioxidant enzyme heme oxygenase-1 Protection of nigral dopaminergic neurons against cell death Reduction of astrocytes and microglia activation and decrease of proinflammatory mediators release in basal ganglia	BV2 microglial cell line MPTP mouse model of Parkinson's disease	Jazwa et al. (2011)

Table 2 continued

Source of GLs	Effects	Subjects	References
Nano-Sulforaphane	Prevention of developmental defects of nervous system induced by PhIP, heterocyclic aromatic amines (HAA) formed by cooking meat at high temperatures	Animal embryonies	Zhang et al. (2021)
4(α -L-rhamnosyloxy)-benzyl ITC (glucomoringin ITC)	Regulation of reticular fibers distribution in connective tissue, nuclear factor (NF)- κ B translocation, I κ B- α degradation, expression of iNOS, and apoptosis	Mouse model of spinal cord injury	Giacoppo et al. (2015a)
4(α -L-rhamnosyloxy)-benzyl ITC (Glucomoringin ITC)	Reduced TNF-alpha release, I κ B-alpha cytosolic degradation/NF κ Bp65 nuclear translocation Decreased markers of inflammation (phospho-ERK p42/44, p-selectin) and oxidative stress (inducible Nitric Oxide Synthase (iNOS), MMP-9)	Rat model of cerebral ischemia/reperfusion damage	Galuppo et al. (2015)
4(α -L-rhamnosyloxy)-benzyl ITC (glucomoringin ITC)	Potent anti-inflammatory activity	Murine Subacute Parkinson's Disease	Giacoppo et al. (2017b)
Allyl-ITC (In lower micromolar range)	Reduced NF- κ B expression Inhibition of c-Jun N-terminal kinase	Murine microglial cells treated with LPS	Subedi et al. (2017)
<i>Eruca sativa</i> seed extract	Reduced apoptosis and production of pro-inflammatory cytokines Reduced COX2 and TLR4/NLRP3 inflammasome expression Restauration of interleukin 10 expression	NSC-34 motor neurons treated with the medium of LPS-treated RAW 264.7	Gugliandolo et al. (2018)

modulation and chemoprevention) attributed to natural ITCs and those of the gas transmitter H₂S can be clearly observed (Martelli et al. 2020b). Naturally occurring H₂S donors, like ITCs, are widely investigated since their safety profile is usually greater than many synthetic donors. The limitations to their use can be the structural amenability to chemical transformations (e.g., during the isolation of these compounds from the plants), the poor water solubility, and the generation of by-products after H₂S release. Thus, in recent years, ITCs have been used as scaffold for the synthesis of new stable compounds or have been combined with other drugs to obtain multitarget molecules with improved anti-inflammatory, antioxidant, and neuroprotective effects (Sestito et al. 2019).

In the context of pain pharmacology, it is important to point out the dual role of H₂S, which essentially depend upon the doses: a low dose contributes to reduce pain, while a high dose elicits pain. Consistently, it has been observed that, by employing slow releasing H₂S agents or low dose of H₂S, attenuation of pain can be achieved. Several mechanisms account

for this pharmacological effect of H₂S, such as the reduction of inflammation, CGRP and oxidative stress (Guo et al. 2020), as well as the activation of KATP and Kv7 channels (Mannelli et al. 2017; Martelli et al. 2013). Indeed, Kv7 voltage-gated potassium channel subunits have been demonstrated to be responsible for most of the H₂S-mediated effects of ITCs (Martelli et al. 2014; Schleifenbaum et al. 2010). Selectively blocking Kv7 channels with XE-991 (Wang et al. 1998) has been shown to cause primary sensory neurons hyperexcitability (Zhang et al. 2019). Accordingly, the activation of Kv7 channels (Miceli et al. 2008) exerted neuroprotective effects against chemotherapy-induced neuropathy (Abd-Elseyed et al. 2015; Nodera et al. 2011) and decreased osteoarticular and neuropathic pain (Brown and Passmore 2009; Li et al. 2008a). In 2018, Di Cesare Mannelli et al. reported that XE991 pre-treatment fully counteracted the pain-relieving effects of ITCs and NaHS, administered by either the systemic or intracerebroventricular route, in a mouse model of oxalipatin-induced neuropathy. Accordingly,

electrophysiological *in vitro* experiments revealed that Kv7.2/3 heteromeric currents, involved in the regulation of pain transmission through the spinothalamic pathway (Wang et al. 1998), can be concentration-dependently activated by H₂S-releasing ITCs. In the same animal model of neuropathy, the dose-dependent rise of pain threshold mediated by both glucoraphanin (GL) and sulforaphane (ITC) was fully prevented by the simultaneous administration of haemoglobin and XE991 (Lucarini et al. 2018b). Likewise, in a model of diabetic neuropathy induced by streptozotocin, the administration of a bio-activated *Eruca sativa* defatted meal or its main GL, glucoerucin, caused a dose-dependent pain-relief dependent upon H₂S release and Kv7 activation (Lucarini et al. 2019b). Kv7 channel thus emerged as the main targets responsible for the antinociceptive activity of GLs and ITCs.

H₂S pronociceptive effect, which occurs in the case of a massive bioavailability of this gas transmitter, seems instead mediated by the positive modulation of T-type calcium channels, voltage-gated sodium channels, as well as TRPA1, TRPV1 and TRPC6 channels, the upregulation of spinal NMDA receptors and the sensitization of purinergic receptors (Guo et al. 2020). A series of mechanisms which are likely less sensitive to H₂S, needing a great stimulus to be activated. The bell-shaped dose-dependence showed by ITCs (Mannelli et al. 2017) likely reflect the complex pharmacokinetics and pharmacodynamics of H₂S-releasing molecules (Ahmad et al. 2016; Szabo et al. 2014).

Slow H₂S-donors, long-lasting generating low concentrations of H₂S, such as GYY4137, are preferred choice in the treatment of disorders like chronic pain (Li et al. 2008b). GLs, being hydrolysable *in vivo* (Fahey et al. 2015, 2019), can be assimilated to a slow H₂S-donor and effectively employed for pain management. Indeed, though ITCs are considered responsible for the pain relief, GLs show a better kinetic profile since they are usually more stable than ITCs, especially in solution (Fahey et al. 2017), while *in vivo* they can mediate a slow release of ITCs and consequently an even slower and longer release of H₂S. Although the role played by H₂S in the acute antinociceptive effect of GLs and ITCs appear to be clear, the involvement of H₂S in the neuroprotective efficacy of these phytochemicals has not been ascertained yet. Anyway, sulphur-containing natural compounds, proved to be neuroprotective because of their

direct and indirect anti-inflammatory and antioxidant properties, including the scavenging of radicals, the down-regulation of microglial-derived inflammatory mediators and cytotoxic effects in astrocytes (Venditti and Bianco 2020). Altogether these findings support the hypothesis that a controlled release of H₂S can contribute also to the neuroprotective efficacy showed by GLs and ITCs against chronic pain.

Other possible mechanisms involved in GLs-mediated anti-hyperalgesic effects

Increasing evidence attested the effectiveness of GLs and ITCs intake in preventing cognitive deficits induced by chemical agents, such as phencyclidine (Shirai et al. 2015), by traumatic brain injury or by stressful stimuli (Zhao et al. 2005b). In a mouse model of irritable bowel syndrome, the administration of an extract of *Camelina sativa* var. *Madalina* defatted seeds, rich in sinapine, GLs, and flavonol glycosides, resulted able to counteract oxidative stress in both brain and bowel tissues, as well as the concomitant behavioural alterations (Cojocariu et al. 2020). Sulforaphane, the ITC derived from glucoraphanin, has been reported to be able to counteract the damage caused by traumatic brain injury and enhancing cell survival by simultaneously reducing lipid peroxidation, decreasing blood brain barrier permeability, and increasing the expression of aquaporin 4 channels (Dash et al. 2009; Sajja et al. 2018; Zhao et al. 2005c). The latter effect appears particularly interesting in the context of glymphatic system, highly active during sleep, which is responsible for clearing away waste from the brain and essential for maintaining brain immune homeostasis across the lifespan (Eugene and Masiak 2015; Plog and Nedergaard 2018). A recent review suggested GLs and the potential modulation of aquaporins activity as new approach to improve the quality of life in women suffering from endometriosis (García-Ibañez et al. 2020). Thus, though the role of aquaporins in pain need to be further investigated, the positive effect of ITCs on aquaporins might empower their anti-inflammatory and neuroprotective profile, strengthening the rationale of their use for chronic pain management.

Yet, recently, cGMP-dependent protein kinase I, already known to contribute to H₂S-mediated vasorelaxation (Bucci et al. 2012) and localized also in nociceptors, was found to be critically involved in the

mechanisms of central sensitization and neuropathic pain establishment (Wang et al. 2021). Albeit preliminary, these observations pave the way for new studies aimed at understanding if cGMP system modulation might be another hidden mechanisms behind the beneficial effects of natural occurring H₂S donors, like GLs, on pain.

Finally, it is important to mention the role played by GLs and ITCs in the maintaining of intestinal microbial homeostasis. Indeed, GLs can be metabolised in vivo into ITCs by members of the human gut microbiome that regulate host epigenetics (Li et al. 2011; Shock et al. 2021). Vice versa, these phytochemicals are active modifiers of gut microbial communities (Ding et al. 2020; Kaczmarek et al. 2019b). Broccoli consumption in humans has been shown to increase intestinal Firmicutes and decrease of Bacteroidetes, respectively (Kaczmarek et al. 2019a). These bacterial phyla together represent more than 90% of the total gut microbiota (Qin et al. 2010) and the alteration of Firmicutes/Bacteroidetes ratio, has been associated with several pathologic conditions (Guo et al. 2019; Li et al. 2020; Minerbi et al. 2019b; Pittayanon et al. 2019; Zeber-Lubecka et al. 2016). At the same time, human gut microbiota conditioned by a *Brassicaceae* enriched diet contributes to increase GLs bioavailability (Sikorska-Zimny and Beneduce 2020). GLs hydrolysis products, like sulforaphane and benzyl ITC, have significant antimicrobial activity against Gram-positive and Gram-negative bacteria isolated from the human intestinal tract, and might help in preventing pathogen overgrowth once administered in patients (Aires et al. 2009). Moreover, in vitro it has been observed that broccoli leachate media can favour the growth of lactic acid bacteria, which implies an increased production of lactate and short-chain fatty acids, demonstrating a prebiotic effect for GLs and ITCs (Kellingray et al. 2021). Interestingly, intestinal dysbiosis co-occurs with the development of several painful diseases. Among them there are either gastrointestinal disorders, namely irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBDs), or extra-intestinal diseases such as fibromyalgia, metabolic syndrome, rheumatic diseases, allergic and atopic disease, and neuropsychiatric disorders (D'Amato et al. 2020; Defaye et al. 2020). Accordingly, gut microbiota has been reported to regulate not only visceral sensitivity, but also musculoskeletal and neuropathic pain (Boer et al. 2019; Dekker Nitert

et al. 2020; Ding et al. 2021; Minerbi et al. 2019a). Considering that changes in the composition and metabolism of microbiota almost inevitably cause alterations in the mechanisms mediating pain signalling (Lucarini et al. 2021a; O'Mahony et al. 2017; Pusceddu and Gareau 2018), the role played by GLs and ITCs in maintaining the microbial homeostasis might represent an additional mechanism in the regulation of pain disorders.

GLs bioavailability

The bioavailability of GLs or their active metabolites depends on several parameters grouped as follows: (1) the concentration of GLs in the plant; (2) the conditions of storage and processing of the raw material and the stability of myrosinase; (3) GLs and derivatives peculiar physico-chemical properties; (4) gastrointestinal transit and microbiota fermentation (Banerjee et al. 2014; Fernández-León et al. 2017; Holst and Williamson 2004a; Jones et al. 2006).

GLs and ITCs found in Brassica-vegetables are subject to a variety of changes during food processing and the analysis of degradation products is often challenging (Barba et al. 2016). During the fermentation, commonly used for white cabbage in Germany ("Sauerkraut"), the major breakdown products are aliphatic ITCs, and ascorbigen (Hanschen et al. 2014). In general, thermal degradation leads to the transformation of GLs predominantly to nitriles through several chemical mechanisms. ITCs are instead severely affected by thermal treatment, and decompose further to a variety of volatile and non-volatile compounds. Anyway, GLs can be differently degraded according to the heat treatment which is employed. For example, mild heat treatment inactivates the epithiospecifier protein, without altering myrosinase activity, thus increasing ITCs formation (Jones et al. 2010). Longer or high temperature (80 °C) heat treatment also inactivate myrosinase, (Björkman and Lönnerdal 1973; Ghawi et al. 2012), resulting in a higher content of GLs and a consequent decrease amount of free ITCs (Barba et al. 2016). Boiling can severely affect the levels of GLs (Nugrahedhi et al. 2015; Verkerk et al. 2009), causing GLs losses of 5–75%, varying as a function of each GL structure (indole GLs are thermally less stable than aliphatic ones) (Hanschen et al. 2012) and the context in which it is found. Indeed, besides heat, several other

conditions, such as pH value, plant matrix, Fe^{2+} ions, vitamin C, other antioxidants, or the water content, strongly affect the susceptibility and the pathway of the reaction, leading to a wide range of metabolites, whose biological activities are mostly unknown (Hansch et al. 2014). Hence, the evaluation of the most appropriate cooking method should be considered an important factor to preserve the beneficial effects of *Brassicaceae*, which are mainly due to GLs and ITCs content (Baenas et al. 2019).

When raw cruciferous are consumed, GLs are hydrolysed by myrosinase in the upper gastrointestinal tract to different metabolites (ITCs, nitriles, oxazolidine-2-thiones, and indole-3-carbinols) while, when cooked cruciferous are ingested, GLs can transit to the colon where they are hydrolysed by the resident microflora (Dinkova-Kostova and Kostov 2012). At this level, based on inter-individual microbial diversity, a wide range of metabolites can be generated (Barba et al. 2016). Interestingly, the frequent intake of *Brassica* sp. vegetables favours the growth of GLs-hydrolysing bacteria within the gut (Angelino and Jeffery 2014). Although the largest fraction of GLs is metabolized in the gut lumen after ingestion, their absorption mostly occurs in the small intestine, where, a percentage is absorbed by the epithelium (Angelino and Jeffery 2014). Although several studies demonstrated that GLs can be absorbed in their intact form, few researches were carried out to study the absorption mechanism of intact GLs and the fate of their major degradation products (Budnowski et al. 2015). Overall considering their chemical features, unmodified GLs can scarcely reach the human tissues, whereas their breakdown products, such as ITCs, are more easily distributed throughout the body (Holst and Williamson 2004b), since they can cross the intestinal epithelial barrier by passive diffusion. After they are absorbed, GLs metabolites can have very different fates, they can be metabolized further in the enterocyte or secreted back into the gut lumen, alternatively, they can pass into the plasma (Angelino and Jeffery 2014). Before passing into the plasma, ITC can be also conjugated with the thiol group of the glutathione system. The resulting GSH-ITC conjugate can dissociate again at low glutathione plasma concentration or get enzymatically cleaved to release the free, and biologically active ITC (Shakour et al. 2021) which can passively cross cell membranes either as free compounds or as cysteine-bound derivatives (Holst

and Williamson 2004b). Ye et al. demonstrated that broccoli sprouts contained ITCs are quickly distributed in the organism, from which they are eliminated following a first-order kinetics (Holst and Williamson 2004b; Ye et al. 2002). These chemical and pharmacokinetic properties, combined with evidence about the acute pain relief mediated by GLs and ITCs (effect peaking between 15 and 45 min), indicates that through the systemic blood circuit most of the ingested ITCs can adequately reach the different districts sites of pain regulation in the body where they can exert their beneficial effects. Moreover, the above-mentioned binding to plasmatic GSH might contribute to the slow and sustained release of ITCs, and consequently H_2S , at the target sites, supporting their protective effects on tissue physiology.

Other important discriminants in compounds bioavailability are the inter-individual variability which can occur in the response to the xenobiotics (Cartea and Velasco 2008) and the peculiar features of the GLs employed. For instance, the activity of GLs-metabolizing microbiota can vary a lot from individual to individual and not all the glucosinolates can be effectively hydrolysed by the same gut bacteria (Lucarini et al. 2018b, 2019a). It is thus clear the importance to guarantee an adequate intake of these phytochemicals by a correct manipulation of raw materials and an optimized formulation of final preparations.

GLs-analgesic drugs interactions

Little is known about the positive or negative relationships occurring between GLs, their metabolites, and analgesic drugs, though it is an important aspect to consider when developing therapeutic plans. ITCs can potentially interfere with the pharmacokinetics of several molecules which are substrate of the ATP-binding cassette transporters, including most multidrug resistance proteins (Telang et al. 2009). Sulforaphane can also compete with the metabolism of other drugs, since it is a substrate of phases I, II, III enzymes. Moreover, sulforaphane turned out to be a potent inducer of phase II enzymes and regulator of cytochrome P-450 expression and function (Zhou et al. 2007). These mechanisms support the preventive effects which were attributed to this natural ITC in degenerative diseases. On the other hand, the modification of the above-mentioned detoxification

systems might alter the bioavailability and bioactivity of concomitantly administered drugs (Fimognari et al. 2008; La Marca et al. 2012; Telang et al. 2009). The in vivo pharmacodynamic interactions between GLs and other analgesic drugs are almost unknown. Allyl-ITC has been reported to prevent the hepatotoxic effects caused by acetaminophen (Kim et al. 2020; Lim et al. 2015), while sulforaphane resulted able to improve *H. pylori*- and NSAID-induced gastrointestinal symptoms in mice and humans (Yanaka 2017; Yanaka et al. 2009). Likewise, also nonsteroidal anti-inflammatory drugs formulated to release H₂S in vivo demonstrated to be less gastro-harmful than the parent drugs due to the positive effects showed by this gaseous mediator on the gastrointestinal tract (Dief et al. 2015; Fiorucci et al. 2007).

Noteworthy, sulforaphane can cause an augmented expression of opioid- μ receptor (Wang and Wang 2017), enhancing morphine analgesic efficacy (Ferreira-Chamorro et al. 2018; Fu et al. 2021; Redondo et al. 2017) and therefore reducing the dose required to maintain the analgesic effect during long-term therapies. Moreover, H₂S released by ITCs has been reported to inhibit opioid withdrawal-induced pain sensitization and to attenuate opioid dependence (Yang et al. 2014a, b).

Although the positive or negative implications of administering GLs together with other drugs need to be further investigated, the association with GLs might not only empower the efficacy of classical analgesics, but also prevent some of their side effects, improving the adherence of patients to the therapy.

GLs toxicity

Ruminants and horses which ingested high levels of allyl-ITC displayed an irritation of the gastrointestinal mucosa accompanied by abdominal pain and cramps (Taljaard 1993). Anaemia is another possible adverse effect resulting from an excessive consume of *Brassicaceae* (Herr and Büchler 2010). An exaggerated intake of vegetables and/or seeds from the *Brassicaceae* family, as well as of high doses of GLs, can affect the functioning of thyroid, liver and kidney, can reduce the growth as well as the reproductive performance, but very rarely cause death (Tripathi and Mishra 2007). Nitriles originating from an alternative metabolism of GLs instead turned out to be hepatotoxic. However, no significant or consistent

abnormalities in liver and thyroid function have been observed supplementing mice diet with different sprout extracts in therapeutic quantities. Supporting GLs safety, it has been demonstrated that broccoli sprout intake ameliorates cholesterol metabolism and reduces multiple oxidative biomarkers without causing side effects (Herr and Büchler 2010).

Conclusions

The wide spectrum of benefits, accompanied by a good bioavailability and very limited side effects, gives a high degree of clinical translatability to GLs-based products. Moreover, the efficacy and versatility shown by these phytochemicals in the treatment of different painful conditions, together with the possibility of administering them as food supplements, encourages their use for the treatment of chronic pain in patients.

Acknowledgements This research was supported by the Italian Ministry of Instruction, University and Research (MIUR), and by the University of Florence.

Funding The authors have no relevant financial or non-financial interests to disclose.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Consent to participate Not applicable.

Consent for publication Not applicable.

Ethical approval Not applicable.

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