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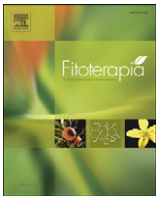

Review

Silymarin and its constituents in cardiac preconditioning

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ABSTRACT

Silymarin, a standardised extract of *Silybum marianum* (milk thistle), comprises mainly of silybin, with dehydrosilybin (DHSB), quercetin, taxifolin, silychristin and a number of other compounds which are known to possess a range of salutary effects. Indeed, there is evidence for their role in reducing tumour growth, preventing liver toxicity, and protecting a number of organs against ischemic damage. The hepatoprotective effects of silymarin, especially in preventing *Amanita* and alcohol intoxication induced damage to the liver, are a well established fact. Likewise, there is weighty evidence that silymarin possesses antimicrobial and anticancer activities. Additionally, it has emerged that in animal models, silymarin can protect the heart, brain, liver and kidneys against ischemia reperfusion injury, probably by preconditioning. The mechanisms of preconditioning are, in general, well studied, especially in the heart. On the other hand, the mechanism by which silymarin protects the heart from ischemia remains largely unexplored. This review, therefore, focuses on evaluating existing studies on silymarin induced cardioprotection in the context of the established mechanisms of preconditioning.

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Contents

Abbreviations: AC, adenylyl cyclase; ALDH, aldehyde dehydrogenase; ANT, adenine nucleotide transporter; AR, adrenergic receptor; ARE, antioxidant response element; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; COX, cyclo-oxygenase; CsA, cyclosporine A; DAG, diacylglycerol; DHSB, dehydrosilybin; EGF, endothelial growth factor; EGFR, EGF receptor; FGF, fibroblast growth factor; GSK, glycogen synthase kinase; HIF, hypoxia induced factor; HUVEC, human umbilical vein endothelial cell; IP3K, inositol phosphate 3 kinase; IPC, ischemic preconditioning; IR, ischemia reperfusion; MMP, matrix metaloprotease; mPTP, mitochondrial permeability transition pore; mTOR, mitochondrial target of rapamycin; PDE, phosphodiesterase; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; ROS, reactive oxygen species; SIRT, silent information regulator two ortholog; VDAC, voltage dependent anion channel; VEGF, vascular endothelial growth factor.

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1. Introduction

Silymarin, a well known multicomponent extract from the seeds of the milk thistle (*Sylibum marianum*), has been used for the treatment of various ailments, mainly those of the liver, for over two thousand years [1]. Interest in this venerable remedy has not been lost with the advent of the systematic scientific approach and modern biochemical methods, and there are now over four hundred clinical trials using silymarin or its components for liver related diseases alone [2]. In this day and age, silymarin is available as an extract from several major suppliers, each with its own standard composition, which varies dramatically between suppliers and appears to depend on variety and growing condition of the crop [3–5]. Typically, silymarin contains around 50% silybin, 20% silychristin, 10% silydianin, 5% isosilybin and between 10 and 30% of a typically unidentified organic polymer fraction formed from the above compounds. Additionally, a minor fraction of other flavanols including 2,3-dehydrosilybin (DHSB), quercetin, taxifolin, kaempferol and others is present [5,6]. Some of the constituents, including silybin, are present as a mixture of stereoisomers with contrasting biological activities [7,8] (Fig. 1). It is understandable therefore, that small changes in the chemical composition of the extract can have a strong influence on its biological activity. On the other hand, this is largely irrelevant when working with the purified, individual components of silymarin. It should be noted that as a consequence of consisting of a number of bioactive compounds, silymarin does not have a single molecular target. Indeed, many of its components, as will become apparent from the discussion below, target more than one enzyme or process. Whilst this can be viewed as a pharmacologist's nightmare, the same pharmacologist may find that it can also become a treasure trove of interesting medicinal compounds and precursors. The milk thistle would serve well for this purpose, owing partially due to its wide range and ease of cultivation.

It is understandable, therefore, that more and more attention is being devoted to the possible protective effects of silymarin on organs besides the liver. As such studies examining protection by silymarin against ischemic damage to kidney, liver, brain and heart have emerged. This is most likely tied to the discovery, and more recently improved understanding, of pre- and post-conditioning. Applicable to tissue that has been subject to ischemia, these closely related biological phenomena prevent a large part of the damage that occurs upon its reperfusion. Whilst preconditioning must be applied during the early window, at least 24 h prior to ischemia, or the late window around 30 min prior to ischemia, post-conditioning can be applied immediately upon reperfusion. Given the unpredictable nature of infarcts, post-conditioning is undoubtedly more valuable as a treatment. Preconditioning, on the other hand, could be availed of when ischemia can be anticipated, for example during surgery or transport of organs [9,10]. The most common, and most clinically relevant, examples of this kind of injury are the heart and brain, where ischemic events manifest themselves as heart attacks and strokes respectively. Arguably, due to the increased window for treatment, pre- and post-conditioning of the heart makes a better example. Both pre- and post-conditioning can be induced either by a series of brief ischemia-reperfusion cycles, in which case they are known as ischemic pre- or post-conditioning (IPC), or by pharmacological agents, in which case they are known as pharmacological pre- or post-conditioning. The former was discovered in 1986 [11] using an open chest dog model, whilst the later arguably in 1984 [12]. Whilst IPC is the better known of the two, pharmacological preconditioning is probably more applicable in practice, as well as serving as a useful tool for the study of the mechanisms involved in IPC.

2. Preconditioning and silymarin

Following occlusion of the blood supply, ischemic tissue will eventually die by necrosis (curiously the 1986 study had already established a limit for the length of ischemia which preconditioning can protect against [11]). It follows that reperfusion became the main form of intervention for myocardial infarction. This led to the discovery of ischemia reperfusion (IR) injury of the heart, which occurs, as the name suggests, when following a prolonged period of ischemia, blood supply is restored to the ischemic tissue, paradoxically causing a rise in cell death. This is proposed to occur because the kick-starting of respiration, in cells where most of the ion gradients have all but collapsed, sets up the perfect conditions for the opening of the mitochondrial permeability transition pore (mPTP) and the subsequent induction of apoptosis. In accordance with this model, ischemic cells rapidly become hypoxic and switch to glycolysis for their source of adenosine triphosphate (ATP) hence becoming acidified. At the same time, levels of reactive oxygen species (ROS) increase and levels of ATP drop along with the activity of the Na^+/K^+ ATPase. Due to the increased proton concentration, i.e. intracellular acidification and reduced activity of the Na^+/K^+ ATPase, the Na^+/H^+ exchanger causes an influx of Na^+. This reverses ion-flux through the Na^+/Ca^{2+} antiporter, increasing the intracellular concentration of Ca^{2+}. Under normal conditions this increase in ROS and Ca^{2+} would be sufficient to open the mPTP and induce apoptosis, however, as low pH inhibits mPTP opening, apoptosis does not occur in ischemic cells. Instead the damage occurs upon reperfusion, when the mitochondrial pH begins to normalise with the restoration of the mitochondrial H^+ gradient and all the conditions for the opening of the mPTP have been met [13,14].

Pre- and post-conditioning, must therefore function either by reducing calcium concentrations in the cells [15–17], limiting over-production or accumulation of ROS or increasing the mPTP threshold [13,18]. In fact it has been shown that pharmacological opening of mPTP with atractyloside prevents preconditioning [19–21], whilst preventing this opening with cyclosporin A (CsA) induces preconditioning in rabbit hearts [20]. The latter prevents the binding of Cyclophilin D [22], whilst the former is a direct inhibitor of the adenine nucleotide transporter (ANT) [23]. Rasola et al. [18] suggest that glycogen synthase kinase 3β (GSK3β) and protein kinase Cε (PKCε) may be responsible for the phosphorylation and hence modulation of mPTP components. It appears that when phosphorylated and hence inhibited by PKCε, GSK3β shifts from the voltage dependent anion channels (VDAC) to ANT binding [24].

This coincides with reduced VDAC phosphorylation and may be central to pre- and post-conditioning [25] as there is evidence that GSK3β inhibition is a central and crucial step in

preconditioning [26–28]. Whilst there are studies that challenge this notion in favour of the model where GSK3β is a marker of preconditioning [19–21,29–31], the evidence for the central role of PKCε in preconditioning is solid [27,29,32–41]. It is therefore possible that other targets of PKCε, such as respiratory chain components or aldehyde dehydrogenase 2 (ALDH2), may be responsible for raising mPTP threshold [42–44]. PKCε itself can be activated by an increase in ROS [45], DAG [36], or by phosphorylation by Erk or protein kinase G (PKG) [15,38,46–50]. In turn, pharmacological preconditioning can be achieved through a number of receptors which are known to activate Erk, PKG and phospholipase C (PLC). Thus, as summarised in Fig. 2, it has been established that stimulation through adrenergic, ouabain, acetylcholine, opioid, bradykinin, oestrogen and adenosine receptors triggers preconditioning [38–40,50–63]. Taking into account the number of receptors and downstream components involved in pre- and post-conditioning, along with the number of their possible interactions of the main components of silymarin, the scope of any attempt to pin down the mechanism responsible for silymarin's cardioprotective activity becomes apparent. Silymarin, and silybin in particular, is known to protect a number of organs, including brain, liver, kidney and the gastrointestinal tract. Silymarin's hepatoprotective properties are especially well researched, with over two hundred clinical trials [2].

In addition, several studies have investigated the potential of silymarin in protecting gastric mucosa [64], liver [65], kidney [66,67] and brain against IR injury [68,69]. Curiously, unlike Wang et al. [68], Hou et al. [69] found that whilst

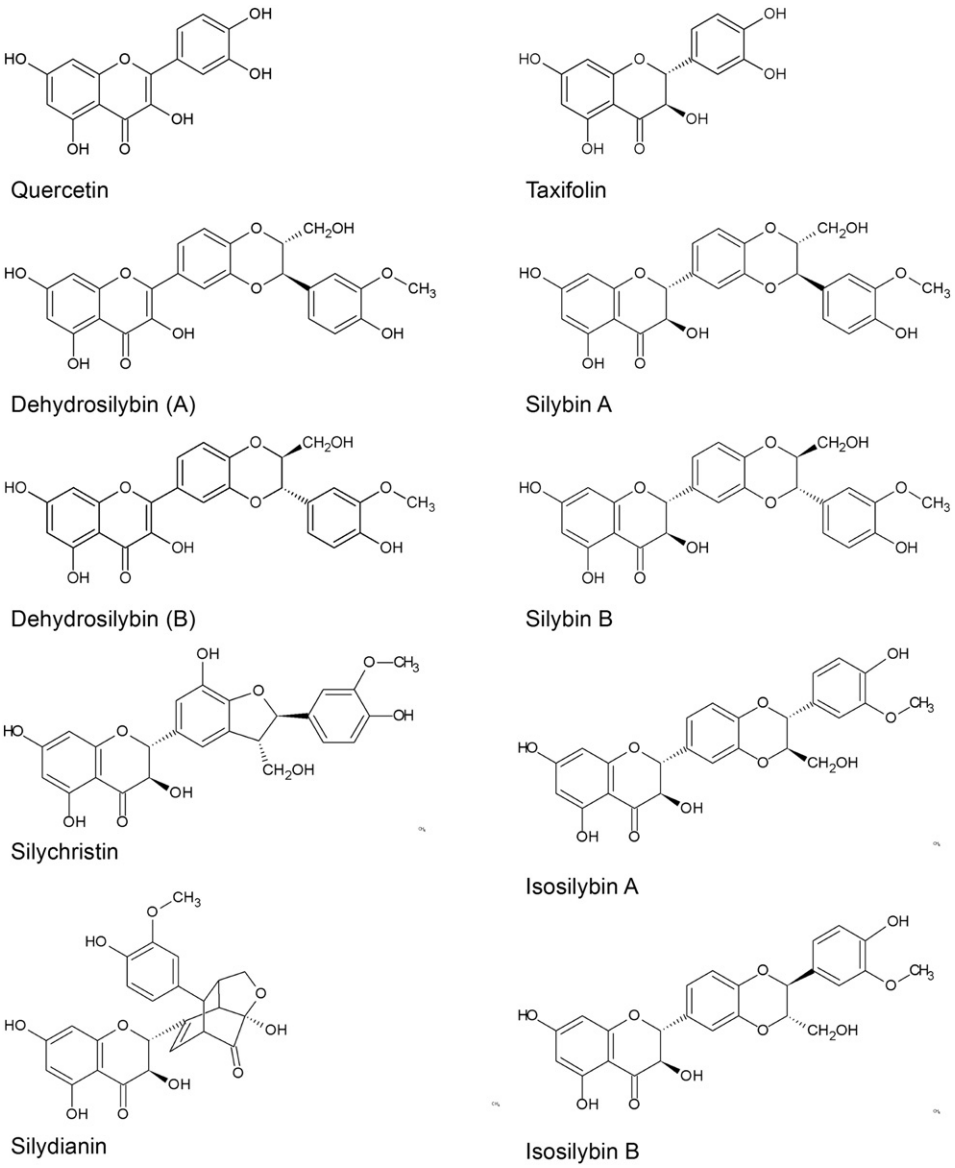

Fig. 1. A schematic diagram of several of the more important components of silymarin; taxifolin, silybin, isosilybin, quercetin, dehydrosilybin, silychristin and silydianin.

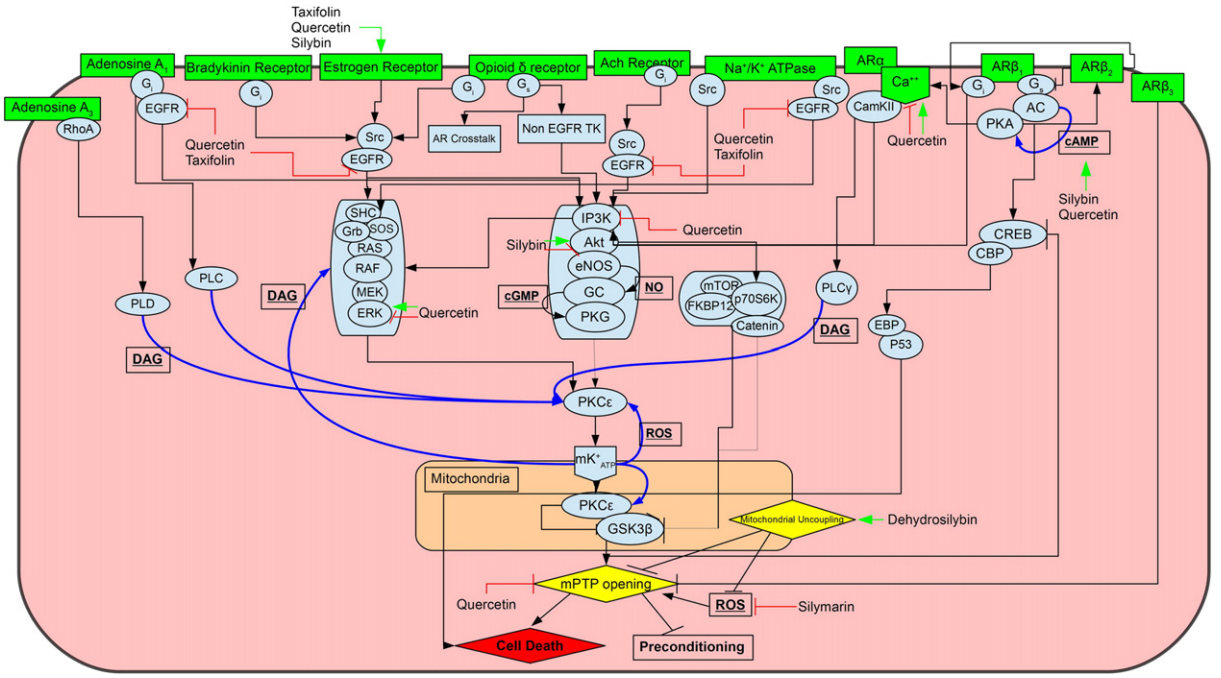

Fig. 2. Diagram outlining the pathways of preconditioning, as evidenced from various models of preconditioning, and the possible interaction points for the components of silymarin that are summarised in this paper. Black arrows indicate interactions between components of the pathways, with blue curly arrows indicating signal transduction by second messengers. Green arrows and red stubbed arrows indicate potential interaction by silymarin's components.

silymarin pretreatment afforded rat brains a certain level of protection against IR injury, silybin did not. This leads to the fascinating possibility that components of silymarin besides silybin, are primarily responsible for the cardioprotective effects of silymarin. As we point out in the Introduction section (vide supra) it is unlikely that silymarin as a multicomponent extract has a single molecular target. This is exemplified by studies whose findings highlight quercetin's ability to protect various tissue types against IR Injury [70–75]. To add to this, a number of studies examined taxifolin's cardioprotective effects in diabetic cardiomyopathy [76] and its ability to precondition against cerebral ischemia [77]. As quercetin and taxifolin are relatively minor components of silymarin, the importance of their contribution to preconditioning by silybin remains unclear. Furthermore, unlike resveratrol, whose cardioprotective effects are known to be mediated via the activation of SIRT1 and inhibition of cyclo-oxygenase (COX) [78–80], it is not quite so clear-cut as to which of silymarin's components could be responsible for the protective effects of the extract. If anything, the situation in the field is somewhat similar to that of cardioprotection by garlic, where it is thought that the effect is attained by a mix of anti-oxidant activity, COX inhibition and potentiation of H_2S signalling [81–86], but definitive proof is lacking, probably because of the complexity of the biochemical cocktail.

There are additional studies evidencing silymarin's action as a cardioprotective agent. The first was a simple IR study in rat hearts, published in 1992 that found silybin to cause a modest reduction in infarct size [87]. This study went largely unnoticed, probably due to language barriers, and the next study on the cardioprotective properties of silybin was not published until fifteen years later. In this study Rao and Viswanath [88] used in vivo rat infarct models to test the effect of week-long feeding of various doses of silymarin on IR injury, using both biochemical markers and infarct size measurements. Silymarin feeding was found to cause a dose dependent decrease in infarct size, lipid peroxide level and glutamate oxaloacetate transaminase levels whilst increasing glutathione transferase and catalase levels [88]. Silymarin was also found to normalise levels of biomarkers of Adriamycin cardiotoxicity, which are elevated by this highly toxic chemotherapeutic agent [89]. Since whole silymarin was used in both studies, it is unclear which of its components was responsible for the cardioprotective effect [88,89]. As the damage associated with the use of Adriamycin is thought to be caused by free radicals, the authors of the study suggested that the antioxidant properties of silymarin are likely responsible for its cardioprotective effect in this instance, although reduction of mPTP sensitivity to free radicals via the various preconditioning pathways remains an equally valid, if untested, possibility [89]. In an investigation of preconditioning by quercetin in an open chest rat model, Jin et al. [72] found a reduction in the levels of markers of inflammation and improved functional recovery in quercetin treated rats, but no reduction in infarct size when rats were treated with 1 mg/kg quercetin prior to ischemia. The study did not rule out a reduction in infarct size at higher concentrations of quercetin. In fact, a simulated ischemia study in cardiomyocytes later found that long term quercetin treatment protected the cells against simulated IR injury [75]. It is noteworthy that inhibition of PKCε was found to prevent cardioprotection in this study, as this suggests that quercetin mediated cardioprotection is true preconditioning, rather than simple reduction in the ROS levels due to its antioxidant

activity. Likewise, Wang et al. [68] found an increase in Akt and mTOR phosphorylation in rat models of cerebral ischemia treated with silybin. As Akt phosphorylation should eventually lead to PKCε phosphorylation this re-enforces the hypothesis that the components of silymarin act via the canonical preconditioning pathways. It is also possible that the protective effect is due to reduction of inflammatory damage to the tissue, as a reduction in NF-κB levels was seen in models of stroke [68,69]. Conversely, a number of the studies mentioned above found increased levels of antioxidant levels and decreased levels of oxidative stress upon treatment with silymarin, silybin or quercetin [73,90,91]. At the same time studies of quercetin preconditioning have pointed to reduced inflammation [72], inhibition of MMP [71] and reduced oxidative stress [74], but as none of these studies compared quercetin to well established methods of preconditioning, it is unclear whether these are effects specifically caused by quercetin or are a general trait of preconditioning. There have been no studies to date testing preconditioning or cardioprotection by purified isosilybin, silychristin or silydianin. However, since silychristin and silydianin have been found to offer at least partial protection to cardiomyocytes against anthracycline toxicity and are generally reported to be antioxidants, it is not unlikely that they may also offer protection against IR injury [92–95].

3. Actions of silymarin that may be related to preconditioning

Apart from the finding that PKCε activity is required for quercetin preconditioning, no study has examined the role of other preconditioning pathways in cardioprotection by this compound [75]. On the other hand, a wealth of information about silymarin's components, has been gleaned from biochemical and molecular studies. These studies have shown several promising directions for unravelling silymarin's mechanisms in preconditioning.

There is a strong argument for the involvement of silymarin in the final steps of the preconditioning pathway. Its constituent flavonolignans and flavonols, with the exception of silydianin and silychristin under certain conditions, have been found to act as antioxidants and radical scavengers, which, as reduction in free radical concentrations increases mPTP threshold, may go some way towards explaining their protective effects [95–97]. This is not, however, the whole story, as the concentrations at which these compounds begin to act as antioxidants were quite high and show a dependence on the system used to investigate them [93,95,98]. In the study by Gabrielova et al. [93], DHSB was found to inhibit free radical formation between 10 and 100 μM in cell based and cell free systems, whilst effectively preventing free radical formation at sub-micromolar concentrations in isolated mitochondria. The authors suggest that as well as acting as a free radical scavenger, DHSB acts as an uncoupler, hence preventing mitochondrial free radical generation [93]. Similarly, silydianin and silychristin were found to be mildly pro-oxidative in models of copper induced LDL oxidation [95], but were found to act as antioxidants in a doxorubicin-iron based models of oxidative damage [98], as well as being capable scavengers of phenylglyoxyl ketyl radicals [99]. Curiously, silybin also proved to be a scavenger in this model. Dorta et al. [94] investigated the antioxidant properties of quercetin and taxifolin. Aside from making certain structural deductions, they found quercetin to be both the stronger anti-oxidant and a respiratory chain uncoupler [94]. It should be noted, that working on isolated mitochondria, similar to Gabrielova et al. [93], this group found that DHSB has antioxidant activities at submicromolar concentrations — indirect evidence that the substances interact directly with the biological system. Silydianin and silychristin, on the other hand, may act as pro-oxidants under certain conditions, but this is unlikely to have a significant effect on the overall properties of silymarin in all systems [95]. The dependence of the components' activity on concentration and model system is further highlighted by additional examples. Certain studies have found that at 20 μM and 50 μM, quercetin can preserve cell viability following treatment with H_2O_2 [100,101]. Whilst another study found that at 50 μM quercetin can increase mPTP opening [102]. The possibility that high concentrations of quercetin increase mPTP opening is supported by ANT inhibition by the compound [102]. There is also some evidence that DHSB interacts with ANT and may be transported by the ion carrier, although it is not clear whether the direct effect of this interaction would be to raise or lower the threshold of mPTP [93]. As such, there is evidence that the components of silymarin may exert a concentration dependent effect on mPTP opening, which may either assist or inhibit preconditioning.

Upstream of the mitochondria silymarin's components has been shown to interact with a number of cellular pathways and the receptors. Aside from direct evidence that silybin B (but not the A stereoisomer), taxifolin and quercetin activate ERs [8], which, in and of itself, should be enough to cause preconditioning [62,63,103–106], there are also hints that these compounds may modulate other receptors. Adenosine and Ouabain receptors, for example, both bind adenosine, marking them as potential candidates for interaction with DHSB and quercetin, as the former was found to bind the nucleotide binding domain of proteins [107] and the latter appears to be an inhibitor of ATPases and ANT [108]. There was also a study by Angelone et al. [109] that suggests that quercetin's inotropic and lusitropic effects are directly dependent on adrenergic receptors. This is further supported by a study by Zhou et al. [110], which found silybin to reverse isoproterenol induced damage in a model of cardiac hypertrophy. As adrenergic stimulants such as isoproterenol, which in the long term induce deleterious effects such as hypertrophy, cause ischemic preconditioning in the short term, it is possible that an agent that prevents AR stimulant induced hypertrophy would also antagonise the mechanisms leading to cardiac preconditioning [110]. On the other hand, the modified coupling to G proteins by long term isoproterenol exposure may make the model inapplicable for preconditioning. There is also the possibility that silybin also inhibits ARβ3, which would lead to preconditioning. It should be noted that the study by Zhou et al. [110] did not elucidate whether silybin directly interacts with ARβs or whether more downstream components of the AR signalling pathway were affected. This raises the possibility that this pathway could be targeted by silymarin via the adenosine binding motifs of AC or PKA. In addition, at around 10 μM, quercetin was found to inhibit phosphodiesterase 4 (PDE4) [111], which is one of the PDE isoforms responsible for negative feedback in PKA/cAMP signalling. Curiously inhibition of PDE by silymarin components is not a recent discovery [112,113].

The inhibition of PDE4 and resulting increase in cAMP levels could also explain the result seen by Angelone et al. [109]. This may also shed light on the increased cAMP levels and vasodilation observed in HUVECs (human umbilical vein endothelial cells) and aortic rings respectively [114]. A comprehensive study by Ai et al. [115] on the effects of silybin in cardiac hypertrophy found that the compound inhibited the phenomenon by blocking EGFR phosphorylation, as well as that of the components of the downstream Erk and Akt pathways. GSK3β phosphorylation was decreased when mice were subjected to aortic banding and increased in sham operated mice. Whilst this effect was recapitulated by the Ang II inhibitor SU1428, the deactivation of Akt and Erk pathways is the opposite effect to that observed in preconditioning. Although the experimental model used here was not one of IR injury and therefore might not accurately reflect silybin's effects in IR injury, the study did suggest that silybin is not the component of silymarin responsible for preconditioning. Incidentally, this was not the only study to show reduced Akt phosphorylation and cell growth upon treatment with silybin. In a study even further removed from our ideal model of IR injury, Singh et al. [116] showed reduced proliferation of HUVEC cells and reduced phosphorylation of Akt at Ser 473 and Thr 308 following 48 h treatment with 10–50 mg/ml silybin. Similarly, Deep et al. [117,118] found that in culture models of prostate cancer isosilybin A decreases Akt phosphorylation, whilst isosilybin B increases it. Curiously in this case upregulation of Akt phosphorylation resulted in a reduction in androgen expression, whilst downregulation increased stimulated apoptosis. Likewise, both topical pretreatment with, and feeding of silybin to, hairless mice prior to UV (ultraviolet) carcinogenesis slowed tumour growth, reducing levels of phosphorylated Akt, Erk and Jnk [119]. In another in vitro tumour model, silybin was found to inhibit the activity of hypoxia induced factor 1 (HIF-1) and the p70S6K/mTOR pathway, whilst paradoxically activating Akt [120]. Curiously, the same study found that hypoxia induced vascular endothelial growth factor (VEGF) release was inhibited by silybin. This echoes the investigation of Deep et al. [121], where the growth of prostate cancer xenografts was slowed by silybin and isosilybin with a decrease in angiogenic markers such as VEGF. Whilst these cancer models are far removed from our ideal models of IR injury, they nevertheless demonstrate that the mechanism of action of silybin differs somewhat from what one would expect of a pharmacological preconditioning agent. Another component of silymarin, quercetin was found to preserve cell viability following treatment with H_2O_2, although studies reached opposite conclusions as to the activation of Erk1/2 during these effects [100,101]. This could be a result of the differences in concentrations of quercetin applied and cell lines used; Ishikawa and Kitamura [100] used 20 μM quercetin, whilst Youl et al. [101] showed increased Erk phosphorylation at 50 μM and above. Additionally quercetin reduced expression of Erb2 and 3 (EGFR family) in a HT-29 in vitro model of colon cancer. This was accompanied by a reduction in the activation of the IP3K/Akt pathway. It is unclear, whether findings in this model would translate to a reduction in EGFR expression in cardiomyocytes [122]. Furthermore, in vitro taxifolin was found to alter gene expression from the antioxidant response element (ARE), including the down-regulation of EGF and fibroblast growth factor (FGF), although the exact consequences of this for IR preconditioning are difficult to interpret [3]. Thus, it is difficult to reconcile the models showing down-regulation of Akt signalling with cardioprotective effects of silybin seen by Chen et al. [87] and the neuroprotective effects seen by Hou et al. [69] and Wang et al. [68] (especially as the last study specifically observed up-regulation of Akt signalling). In fact, given the weight of evidence showing silybin to be an antiproliferative agent which reduces Akt activation, we can only surmise that highly nuanced differences in pathway coupling, specific to the different models, are responsible for the effects observed. It is also possible that prior to ischemia, silybin does in fact down-regulate Akt signalling in the models used by Hou et al. and Wang et al. [68,69], with a rebound in Akt activation upon occlusion. Another, somewhat unrelated, possibility mentioned above is that inhibition of the cyclooxygenase pathway by silybin and taxifolin would reduce the damage caused by inflammation [123,124]. In fact silybin and silydianin have been found to reduce production of hydroxyl radicals by polymorphonuclear neutrophils (PMNs) [125]. A further study by Zielinska-Przyjemska et al. [126] suggests that, at least in the case of silydianin, this is due to increased apoptosis of PMNs due to the induction of caspase 3. This fits in with the suggestion that silymarin prevents IR injury, at least in part, by reducing inflammation [68,69].

Compound	Effect	Model(s)	References
Silybin	Cardioprotection	Aortic banding mouse model	[115]
	Preconditioning	Open chest Sprague–Dawley rats,	[87]
		Open chest Wistar rat model	[88]
		Long–Evans rats ischemic stroke	[69]
		BALB/C mouse ischemic tourniquet-gastrocnemius	[91]
		Sprague Dawley rats ischemic stroke	[68]
	Antioxidant	In vitro assays (silybin dihemisuccinate)	[96]
		Copper induced oxidation model (silybin, silydianin, silychristin)	[95]
	PDE inhibitor	In vitro assays, partially purified beef heart PDEs	[112]
	HIF inhibitor	HeLa and HEP-3 cell model	[120]
	GLUT inhibitor	3T3-L1 and CHO cell models	[127]
	ER stimulation	T47D.Luc cell culture luciferase reporter model	[8]
	Hypoxia induced VEGF release	HeLa and HEP-3 cell model	[120]
	Anti-inflammatory	Mouse tail-flick and writhing model	[128]
	Anti-proliferative activity	UV irradiated SKH hairless mice	[111]
	Akt modulation	HeLa and HEP3B culture model (stimulation)	[120]
		HUVEC (inhibition)	[116]

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(*continued*)

Compound	Effect	Model(s)	References
		UV irradiated SKH hairless mouse (inhibition)	[119]
		Wistar rat IR model (inhibition)	[88]
	Antiviral (HCV)	Cell culture and ex vivo models.	[129]
Dehydrosilybin	Mitochondrial Uncoupling	Rat myocyte, mitochondria and in vitro models	[85]
	Antioxidant activity	Ex vivo and microsomal assays	[130]
		Review	[131]
	GLUT inhibition	3T3-L1 and CHO cell models	[120]
	Topoisomerase inhibition	EPI and FIB cell nuclear extracts	[132]
		In vitro topoisomerase assay	[133]
	MDR inhibition	T5-HeLa membrane vesicle preparations	[108]
		Review	[134]
		Sensitive and resistant cancer cell lines	[135]
		MRP1-BHK1 cell lines and membrane vesicle models	[107]
		Plasmodium falciparum strains	[136]
Taxifolin	Anti-inflammatory	Rat paw oedema model	[137]
	EGF/FGF downregulation	HCT 116 cell model	[3]
	ER stimulation	T47D.Luc cell culture luciferase reporter model	[8]
Quercetin	Cardioprotection	Neonatal rat myocytes, simulated ischemia model	[75]
		Open chest Sprague–Dawley rat model	[72]
		Simulated ischemia, embryonic rat ventricular cells	[73]
	Preconditioning	Ischemic kidney Sprague Dawley rat model	[74]
		Ischemic stroke C57BL6 mouse model	[71]
	Vasodilation	HUVECs & aortic rings	[114]
	MPTP opening	Rat kidney cortex mitochondria	[94]
	IP3K inhibition	X-ray crystallography	[138] [139]
	ERK modulation	Aortic banding mouse model. (Inhibition)	[119]
		UV irradiated SKH hairless mouse (Inhibition)	[111]
		SM43 rat mesangial cell culture model (Inhibition)	[100]
		INS-1 cell culture and rat Langerhans islet preparation (Activation)	[101]
	EGFR downregulation	HT-29 colon cancer culture model	[122]
	ER stimulation	T47D.Luc cell culture luciferase reporter model	[8]
	ANT inhibition	Rat kidney cortex mitochondria	[102]
	MDR inhibition	T5-HeLa membrane vesicle preparations	[108]
		Review	[134]
	Ca^{2+} channel modulation	NG108-15 cell model	[140]
	PL-PK inhibition	Mouse Brain Ca PL-PK	[141]
Silychristin	Pro-oxidant	Ex vivo LDL oxidation assay.	[95]
	Antioxidant	DPTT/DPTA ex vivo model.	[99]
		Rat mitochondria/ microsome models, DPPH ex vivo assays.	[98]
		DPTT/ORAC/HORAC/ TEAC/TAC ex vivo model.	[142]
		Huh7.5.1 cell model.	[143]
	Chemoprotection	Rat cardiomyocyte model.	[92]
Silydianin	Pro-oxidant	Ex vivo LDL oxidation assay.	[95]
	Antioxidant	DPTT/ORAC/HORAC/ TEAC/TAC ex vivo model.	[142]
		Rat mitochondria/ microsome models, DPPH ex vivo assays.	[98]
		DPTT/DPTA ex vivo model.	[99]
		Huh7.5.1 cell model.	[143]
	Anti-inflammatory	Isolated human PMNs. Chemiluminescence and O_2•-formation.	[126]
		Isolated human PMNs.	[125]
		Huh7.5.1 cell model.	[143]
	PPARγ downregulation	3 T3-L1 cell model.	[144]
	Chemoprotection	Rat cardiomyocyte model.	[92]
Isosilybin	PPARγ activation	HEK-293 luciferase reporter assays	[145]
	Antioxidant	ORAC/HORAC/TEAC/ TAC ex vivo model.	[142]
		Huh7.5.1 cell model.	[143]
	Anti-inflammatory	Human peripheral blood mononuclear cell model.	[143]
	Antiviral (HCV)	Huh7.5.1 cell model.	[137]
		Cell culture and ex vivo models.	[129]
	Increased Akt Phosphorylation	Isosilybin B — prostate cancer cell culture models.	[118]
	Decreased Akt phosphorylation	Isosilybin A — prostate cancer cell culture models.	[117]

4. Future directions

The hypothesis that silymarin protects tissue against ischemia, as highlighted in this text, is supported by a considerable collection of evidence. However, neither the role that each

component of silymarin plays in the process, nor the timing is entirely clear. To compound this, prevention of IR injury by purified isosilybin, silychristin and silydianin has not yet been tested.

The component responsible could be elucidated by large scale cell culture based simulated ischemia studies, or better small animal studies, designed to compare each of the components side by side, along with the whole extract. At the same time, the mechanisms by which silymarin's components cause cardioprotection should be cross-examined by observing the effects of inhibitors of the classic pathways of preconditioning on silymarin induced cardioprotection. Whilst cell culture models may be suitable for this type of study, they possess several important disadvantages, including differences in metabolism and perfusion and the impossibility of examining the effect of alterations in the immune system by the formulation in question on IR injury. In addition, transgenic mouse models, such as those by Juhaszova et al. [27] or Gomez et al. [28] would be very useful in elucidating whether the effects of silymarin's components are due to true preconditioning or antioxidant/radical scavenging effects. As silymarin's components have been shown to affect markers of inflammation, the use of a model that does not account for the immune system when studying cardioprotection by silymarin may leave the investigators with an incomplete set of conclusions.

As there are numerous studies showing that silymarin is generally safe when studying diseases of the liver [2,146], clinical studies of silymarin or its components in cardioprotection are not beyond the realms of possibility. It is questionable, however, whether a clinical trial that is capable of advancing the field can be designed with the current understanding of the compounds in question.

5. Conclusion

Overall, the evidence seems to indicate that the constituents of silymarin reduce the activity of both the Erk/MEK and IP3K/Akt pro-survival pathways, whose activation is central to ACh, bradykinin, and ouabain preconditioning. At the same time, it makes sense that stimulation of oestrogen receptors, inhibition of MMPs, PDEs, and mitochondrial ROS generation by silymarin's components should facilitate preconditioning. Furthermore the antiinflammatory properties of certain components may also have a role to play in protecting tissue from IR.

As we have highlighted in this review, deciphering the mechanisms of action of silymarin in preconditioning is a fairly involved affair. Summary of available literature shows that silymarin and its components do influence signalling pathways, which are involved in preconditioning. Whilst the major component of silymarin, silybin, is the usual suspect when it comes to these salutatory properties, other, minor components of the extract have also been shown to possess an important cardioprotective activity. With this in mind, it is our belief that the individual components of silymarin constitute a family of compounds worth investigating in relation to ischemia reperfusion. Mechanisms of their action, if properly understood, promise a relatively inexpensive way of broadening the spectrum of pharmacological agents available for treatment of ischemia reperfusion injury.

Conflicts of interest

The authors have no conflicts of interest to declare.

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