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Disposition of Pharmacologically Active Dietary Isoflavones in Biological Systems

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Abstract

Dietary isoflavones, popularly known as phytoestrogens, represent one of the most biologically active classes of flavonoids. Numerous *in vitro*and*in vivo*studies provide convincing evidence regarding their beneficial effects on human health. These isoflavones are increasingly being investigated as potential alternate therapies for a range of hormone-dependent conditions, including cancer, menopausal symptoms, osteoporosis and cardiovascular diseases. However, they exhibit poor oral bioavailability which limits their clinical utility in humans. The reason being, they are substrates of a plethora of enzymes and transporters and undergo extensive conjugative metabolismwhich facilitate their rapid elimination from biological systems. In addition, a number of experimental studies have also revealed that these isoflavones are potent inhibitors of various cytochrome P450 isoforms and transporters which play an important role in the disposition of many commonly prescribed drugs. Thus, there arise chances of observing clinically relevant herb-drug interactions which could sometimes be life-threatening. This review gives a comprehensive understanding of these dietary phytoestrogens with regard to their absorption, biodistribution and the role of enzyme-transporter interplay affecting their disposition in biological systems. Further, the effects of these phytoestrogens on the activity and kinetics of drug metabolizing enzymes and various clinically relevant influx/efflux transporters and the resulting diet-drug interactions have also been discussed.

Keywords: Conjugates; disposition; efflux transporters; enteric recirculation; isoflavones; phase II metabolism

1. Introduction

Nature has always been generous towards mankind, providing remedies and cures to our ailments right from the start of human civilization. The fact cannot be challenged that the majority of blockbuster drugs used nowadays to treat various human ailments and diseases are derivatives of natural products. In recent years, there has been a resurgence of scientific interest in flavonoids due to the association of these compounds with a wide range of health promoting effects. Numerous studies have indicated that flavonoids have anti-oxidant, anti-carcinogenic, anti-viral, anti-inflammatory, anti-osteoporotic and anti-estrogenic or estrogenic activities. Dietary intake of flavonoids has been linked with reduced risk of cancer, osteoporosis, cardiovascular diseases, and other age-related degenerative diseases [1-5]. Flavonoids or vitamin P (vegetable polyphenols) [6] belong to a group of natural substances with variable phenolic structures and are found in fruits, vegetables, grains, barks, roots, stems, flowers, tea, and wine [7]. These polyphenols occur ubiquitously in plants and possess phenyl-1,4-benzopyrone nucleus chemically. These can be further classified as flavones, flavonols, isoflavones, anthocyanins, flavanols, proanthocyanidins and flavanones based on

the degree of oxidation of the oxygen heterocycle [8]. Flavonoids possess many important biological activities including anti-inflammatory, anti-oxidant, anti-cancer *in vitro*. But whether these properties are relevant *in vivo* or not has been a matter of debate owing to their poor absorption (less than 5%) and bioavailability [9]. Flavonoids as natural selective estrogen receptor modulators (SERM) have gained preference as prophylactic agents against postmenopausal bone loss due to their lack of adverse effects that are commonly associated with hormonal replacement therapy (HRT) [10, 11].In an English study, bone mineral density was compared between older women who consumed tea and those who did not. Women in the study who drank tea had higher bone mineral density measurements than those who did not drink tea indicating that the flavonoids in tea might be responsible for the prevention of osteoporosis [12].Isoflavones class of flavanoids has gained much interest recentlywith more than 13700 publications containing "isoflavones" as a key word upon making a search in Medline/NCBI, National Library of Medicine, USA (PubMed) (Search conducted on 16thOctober, 2012). Figure 1 shows the number of research papers published during the last two decades covering various aspects of flavonoids research. This isdue to their relatively better absorption and bioavailability than other flavonoids [13]. They have shown promising anti-cancer, hypocholestrolemic, anti-osteoporoticand anti-oxidant effects [14]. Isoflavones are structurally similar to the naturally occurring estrogens and hence are also known as phytoestrogens.Because of their superimposable structure to that of mammalian estrogens, isoflavones bind to estrogen receptors and act as their partial agonist and antagonist [14]. The potent pharmacological activities have been attributed to the estrogenic properties of these agents [14, 15].

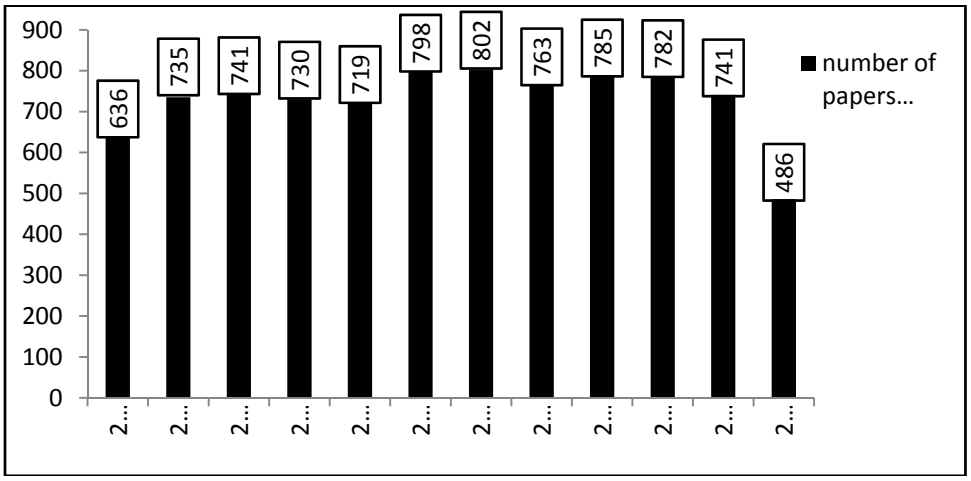

Figure 1: Number of research papers published during the last decade concerning isoflavones research

Phytoestrogens are mainly found in legumes [16]. Soy is the main source of isoflavones genistein, daidzein and glycitein while formononetin, biochanin A are present in high concentrations in red clover in addition to genistein and daidzein [17]. Prunetin is found mainly in the bark of*Prunus emarginata*. Isoflavones occur predominantly in glycosylated form in plants. On absorption isoflavones undergo extensive phase II glucuronidation and sulfation. Thus, it has been proposed that their high metabolism is the reason for their low bioavailability. Furthermore studies determining the factors responsible for the same have revealed that an array of enzymes and transporters maybe involved. The presented manuscript discusses about the enzymes and transporters implicated in the *in vivo* disposition

of these bioactive non-nutrients and also reviews the recent advances in this field, exploring the mutual interactions between isoflavones, drug metabolizing enzymes and efflux transporters. Figure 2 presents the structures of commonly found isoflavones in plants.

(Figure 2: Chemical structure of pharmacologically active isoflavones

Isoflavone	R_1	R_2	R_3	R_4	R_5
Genistein	H	H	OH	H	H
Daidzein	H	H	H	H	H
Biochanin A	H	H	OH	H	Me
Formononetin	H	H	H	H	Me
Glycitein	H	OMe	H	H	H
Prunetin	Me	H	H	H	H

Legend to figure 2: Chemical structures of most pharmacologically active isoflavone phytoestrogenic compounds present in soy and red clover, the richest sources of isoflavones, and oregon cherry.Genistein, daidzein, glycitein and their glycosides conjugates are the major isoflavones present in soy; biochanin A, formononetin and their malonyl derivatives are the major components present in red clover while prunetin is present in *Prunus emarginata*, the oregon cherry.)

2. Absorption

Isoflavones in their natural form are mainly present as glycosides. The glycosides are high molecular weight polar compounds [16]. They need to be first converted into their aglycone forms to be absorbed [18]. Consequently, the absorption of aglycones have been found to be higher and more rapid than their glycosides in humans [19], which seems logical because isoflavones as such are high permeability compounds as can be predicted from their pKa values [20-23]. Studies have shown that the bioavailability of isoflavones is better when fermented soy foods were consumed[24, 25] since these contain the aglycone form. The absorption rate particularly has been found to be greater for aglycones than for the glycones [26] . In a series of comparative studies on genistein, genistin and tofu in isolated rat small intestine, genistein was more efficiently absorbed into the vascular side from lumen, than genistin [27-29]. Consistent with these results, the apical to basolateral transport of genistin was found to be negligible as compared to its aglycone across human intestinal Caco-2 cells [30]. Similar findings were also observed for daidzein and its glycoside [31]. The greater intestinal absorption of genistein as compared to genistin have been reaffirmed *in situ* using perfused rat intestinal model and *in vivo* by portal vein sampling in unanesthetised rat as well [32, 33]. Piskula *et al*. investigated the absorption of genistein, daidzein and their glycosides from stomach in anesthesised rats. They observed that genistein and daidzein were detectable in blood 3 mins after administration of aglycones while they were not detectable even at 10 mins after administration of glycones. The results obtained in pylorus ligated rat model showed that on even on restricting the absorption site to stomach, isoflavone metabolites were detected in plasma on administration of aglycones, daidzein and genistein but not on administration of daidzin and genistin. This further strengthened the postulate that aglycones are absorbed faster and in higher amounts than glycones [34]. However, conflicting results are also available in literature showing that there is no or little difference in the plasma profile

of aglycone and glycoside isoflavones in humans [26, 35]. Setchell *et al*. found that the ingestion of genistein or daidzein glycones and aglycones produced comparable plasma profiles. Infact the systemic bioavailability of the glycosides from dose normalized area under the curve (AUC) was found to be higher. They suggested the protection of the isoflavones from intestinal metabolism due to the presence of sugar moiety as a possible reason for this finding [26]. Zubik *et al*. observed that the AUC, Cmax and Tmax of genistein and daidzein and their glycones were not significantly different in American women when compared for a 48 hour period [35].

Despite the good permeability of the isoflavones, their oral bioavailability is usually low. In the same species, biochanin A was found to be 4.6% orally bioavailable [36]. Apart from gut hydrolysis of the glycosides, theoral bioavailability of isoflavones from aglycones and glycones depends on a number of factors such as food matrix effect, dose, source of isoflavone as well as patient related factors such as age, gender, type of gut microflora, gut transit time *etc*., which have been reviewed in detail by Nielson *et al.*[37].

3. Metabolism

3.1.By gut microflora

Human intestine is inhabited by a vast range of bacteria. These bacteria have been thought to be responsible for breaking down the isoflavone glycosides into their aglycones, the absorbable form. Many of the gut bacteria possess β-glycosidase activity [38, 39]. The first biotransformation reactions experienced by orally administered isoflavones are due these bacterial ezymes secreted by the gut microflora [40]. The glycosides are broken down into their aglycones. The aglycone isoflavones may further undergo demethylation, reduction and ring fission in presence of these bacterial enzymes to produce a number of metabolites. Soy isoflavones are metabolized into Dihydrodaidzein; Equol; 3'-Methoxy-7,4'-Dihydroxyisoflavone; Benzopyran-4,7-diol-,3-(4-hydroxyphenyl); 4-Hydroxybenzoic acid; 2,4-Dihydroxybenzoic acid; 2',4'-Dihydroxyacetophenone; 4-Hydroxyphenylacetic acid; Dihydrogenistein; 6,7,4'-Trihydroxyisoflavone [40-44] (Table 1). Red clover isoflavones, biochanin A and formononetin, are biotransformed into genistein and daidzein, respectively, by human intestinal bacteria [41]. 4-ethylphenol is the major end product of genistein metabolism. S-Equol, the first soy isoflavone metabolite to be identified, is an exclusive product of bacterial metabolism of daidzein [45-49]. It is biologically more active than its precursor, daidzein due to its greater structural similarity to mammalian estrogen [14, 50, 51]. Depending upon the habitual diet and the differences in residing gut flora, two types of population groups have been identified, namely, equol producers (people having >20μg/ml plasma equol concentration) and non equol producers (people having <10μg/ml plasma equol concentration) [52, 53].

The critical role of intestinal bacteria in metabolism of isoflavones is supported by the fact that incubation of isoflavones with human fecal extracts led to the formation of products that were recovered in urine [42-44, 54]. Secondly, isoflavone metabolites equol and O-desmethylangolensin were detected in urine of normal rats but not germ-free rats fed on soy isoflavone rich diet [55, 56]. Thirdly, due to the absence of appropriate bacteria, human infants did not excrete equol when fed soy formula [57, 58]. Lastly, specific fecal bacterial species have been identified,such as Escherichia coli HGH21 and the gram-positive strain HGH6, which metabolize isoflavones to products that have been detected in urine [41, 59-61].

However, recently, increasing importance of human intestinal β-glucosidases has been advocated [31, 32, 55]. The appearance of glucuronidesin plasma just 30 minutes after oral ingestion of soy suggests that isoflavones are absorbed in small intestine [62]. But the gut microflora,that was thought to metabolize glycosides into aglycones, is localized in colon. This indicates that there is an involvement of endogenous mammalian β-glycosidases [63].

Work done by Day *et al*. verified that genistein and daidzein glucosides are indeed metabolized to their respective aglycones by lactase phlorizin hydrolase (LPH), a membrane bound, family 1 β-glycosidase. LPH is a transmembrane protein present on the brush border epithelium of the mammalian small intestine and thus, can act on the glucosides before they are absorbed [64].

3.2.Humancellular metabolism

3.2.1. Phase I metabolism

The remaining aglycone isoflavones may further be metabolized by phase I and phase II enzymes in mammalian cells. Phase I reactions are minor pathways for their metabolism. CYP enzymatic biotransformation primarily involves demethylation and hydroxylation of the aglycones. CYP1A2 has been found to be the major CYP isoform involved in oxidative metabolism of soy and red clover isoflavones [65-69]. Table 1summarizes their major metabolites formed from CYP metabolism.

Table 1: Major metabolites formed gut bacterial biotransformation and phase I (mainly CYP1A2) biotransformation of isoflavones.

Isoflavone	Gut bacterial biotransformation		Phase I metabolism	
	Major metabolite/s	Reference/s	Major metabolite/s	Reference/s
Genistein	Dihydrogenistein	[42]	Orobol (minor contribution from CYP2E1)	[66, 67]
Daidzein	Dihydrodaidzein, Benzopyran-4,7-diol, 3-(4-hydroxyphenyl), Equol	[42]	7,8,4'-trihydroxyisoflavone; 7,3',4'-trihydroxyisoflavone; 6,7,4'-trihydroxyisoflavone (extrahepatic contribution from CYP1A1 and 1B1)	[65, 69]
Glycitein	6,7,4'-trihydroxyisoflavone	[41]	8-hydroxyglycitein; 6-hydroxydaidzein	[70]
Biochanin A	Genistein	[41]	Genistein (minor contribution from CYP2E1)	[68]
Formononetin	Daidzein	[41]	Daidzein; 6,7-dihydroxy-4'-methoxyisoflavone; 7,8-dihydroxy-4'-methoxyisoflavone; 7,3'-dihydroxy-4'-methoxyisoflavone	[68]

From urinary excretion studies, dihydrodaidzein, tetrahydrodaidzein, 2-dehydro-*O*-desmethylangolensin; *O*-desmethylangolensin, equol,*cis*-4-OH-equol, dihydrogenistein, tetrahydrogenistein, 6'-hydroxy-*O*-desmethylangolensin, 4-hydroxyphenyl-2-propionic acid, 4-ethylphenol, 6,7,4'-Trihydroxyisoflavone have been found as the soy isoflavone metabolites excreted [46, 49, 71-73].

3.2.2. Conjugation reactions

Phase II reactions are the most important metabolic reactions for isoflavones. The free aglycone liberated by bacterial β-glycosidases is absorbed into the enterocytes where

extensive glucuronidation occurs, catalysed by the microsomal enzyme UDP-glucuronosyltranferases (UGT) [74]. Subsequently, glucuronides are the main circulating form of isoflavones in plasma [75]. Major conjugation of isoflavones occurs in enterocytes accounting for the high first pass metabolism while liver presents a minor site of metabolism for the part of orally administered phytoestrogens that escape gut wall metabolism [74, 76]. Equol, the major metabolite of daidzein, undergoes similar conjugation [77]. Sulfation, catalysed by sulfotranfersases (SULT), and sulfoglucuronidation are the other important Phase II reactions accounting for metabolism of isoflavones [74, 78, 79]. For prunetin, sulfates are the major conjugates formed [20]. Conjugation occurs primarily at C-7 and C-4' positions forming genistein 4'-O-sulfate, genistein 7-O-beta-D-glucuronide, genistein 4'-O-sulfate 7-O-beta-D-glucuronide from genistein; daidzein 7,4'-di-O-sulfate, daidzein 7-O-beta-D-glucuronide, daidzein 4'-O-sulfate from daidzein and glycitein 7-O-beta-D-glucuronide from glycitein [80-82]. Similar metabolites have also been obtained for red clover isoflavones [22, 36]. UGT1A10, the highly expressed UGT isoform in human colon, and SULT 1A1*2, 1E, and 2A1 are the predominant isoforms catalyzing these reactions [74, 83] although it has been illustrated that isoflavone concentration changes have significant impact on the isoforms' contribution. For genistein, glycitein, biochanin A and prunetin, UGT1A9 and 1A1 were the major contributors at 2.5μM and UGT1A8 and 1A10 at 35 μM suggesting important roles for other UGT isoforms also [84]. SULT1A1 is probably involved in monosulfation while SULT1E1 contributes towards disulfation [83].

4. Role of transporters

Role of transporters is being increasingly recognized in the disposition of drugs. One of the main reasons of low oral bioavailability of drugs has been attributed to the fact that they are substrates of gut efflux transporters which hinders their journey from apical to basolateral side of enterocytes. The ATP binding cassette transporters (ABC) superfamily has been majorly implicated for this. The ABC transporters is the largest family of transmembrane proteins and comprises of more than 49 individual genes in humans. Of these, 3 transporters namely P-glycoprotein (Pgp,encoded by the ABCB1 gene andalso referred to multidrug resistance 1, MDR1), multidrug resistance-associatedprotein 2 (MRP2, encoded by the ABCC2 gene)and breast cancer resistance protein (BCRP,encoded by the ABCG2 gene) have been thought to be primary responsible for efflux of drugs and toxins[85]. Genistein and daidzein have been reported to be substrates for this ABC efflux system [86-88]. Imai *et al*. observed that the basal to apical transport of ^{3}Hlabeled genistein was more in LLC/BCRP cells than LLC/PK1 cells while the opposite results were obtained for its secretion. These differences in secretion and absorption disappeared in presence of fumitremorgin c (a BCRP inhibitor), thus, verifying that genistein is a substrate for BCRP [88]. Studies in BCRP knockout mice prove that BCRP restricts the penetration of genistein through blood-brain, blood-testis and blood-placental barriers, all of which express BCRP. The tissue to plasma concentration (Kp value) was found to be 9.2 and 5.6 fold greater in brain for genistein and daidzein respectively, 5.8 fold greater in testis for both the isoflavones and 1.8 fold greater in fetus for genistein [87]. A study performed on genistein and daidzein in Bcrp1(-/-) mice showed an increase in sulfate and glucuronide conjugates of genistein by 7-fold and 8-fold, respectively, after its intragastric administration as compared to those in wild type mice. Likewise, for daidzein, total AUC (including its conjugates) was increased by 6.9 fold in wild-type versus Bcrp1(-/-) mice, after its intragastric administration. The increased exposure of the aglycones isoflavones to UGTs and SULTs in BCRP knockout mice has been suggested as the reason for this observation [86].

Moreover, the phase II metabolites of isoflavones have also been proposed to be substrates of various transporters. Experimentation on genistein conjugates excretion from enterocytes resulted in an almost complete abolition of genistein sulfate excretion and a 78% reduction in genistein glucuronide excretion into small intestine in BCRP knockout mice as compared to normal mice. This indicates that genistein conjugates are substrates of BCRP/ABCG2[89]. In a similar study, Yang *et al*. found enhanced basolateral excretion of genistein conjugates in Bcrp1-/- mice. On addition of 2 and 20 mg/kg genistein, the AUC0-t of genistein-7-glucuronide (G-7-G), genistein-4'-glucuronide (G- 4'-G), genistein-4'-sulfate (G-4'-S) and genistein-7-sulfate (G-7-S)were increased NA, NA, 10.5, 3.5 fold and 16.2, 9.7, 26.0, 8.4 fold in Bcrp1-/-mice, respectively. To support these observations, further studies were performed on Caco-2 cells. In presence of BCRP inhibitor Ko143, the apical excretion of G-7-G, G-4'-G, G-4'-S and G-7-S across Caco-2 was significantly decreased by 25%, 20%, 45% and 54% ($p<0.05$) at 2 μM genistein, thus verifying the importance of BCRP in intestinal absorption of isoflavone conjugates[90]. In a comparable *in vitro* study conducted in MDCK/Mock and MDCK/Bcrp1 cells, the sulfate conjugate of biochanin A was found to be a substrate of Bcpr1. On addition of biochanin A to MDCK culture media, biochanin A sulfate conjugate, formed by the action of SULT1A1 expressed in MDCK cells, was detected in both apical and basolateral sides with preferential basolateral transport in MDCK mock cells and apical transport in MDCK/Bcrp1 cells[91]. All these findings also point out that subtype Bcrp1 is mainly involved in the efflux of these conjugates [90, 91]. In addition the isoflavone conjugates have also been suggested to be substrates of MRP and organic anion transporter (OAT)[20, 30, 92]. OATs belong to solute carrier (SLC) family of transporters and are encoded by the SLC22 gene family. The basolateral and apical excretion of glucuronide as well sulfate conjugates of genistein, daidzein, formononetin and biochanin A across Caco-2 cells was inhibited by administration of MRP inhibitor, leukotriene C_4 (LTC_4), and OAT inhibitor, estrone sulfate. However, LTC_4 did not inhibit the basolateral and apical efflux of prunetin conjugates and estrone sulfate did not inhibit their apical excretion[20]. Jeong *et al*. found similar results for formononetin conjugates in mouse intestinal perfusion model using MRP inhibitors, LTC_4 and MK-571. The excretion of glucuronide conjugates of formononetin was inhibited by 51 and 76% in upper and lower mouse intestinalsegments in presence of inhibitors while intestinal excretion of sulfate conjugates was modestly decreased by around 30%. Commensurating with these were the results in Caco-2 cells where the apical and basolateral excretion of glucuronide were decreases by 93 and 90% and the apical excretion of sulfate was decreased by 50% [92]. Recently, studies done on isoflavones and their conjugates showed that the 7-O-glucuronides of genistein, daidzein and glycetein were transported specifically by OAT3 while genistein-3'-O-sulfate was predominantly transported by OAT1. OAT3 exhibitedmuch broader substrate specificity and was found to transport all the conjugates including sulfate metabolites [93]. Thus, the ABC class of efflux transporters as well as the OAT transporters hasa major involvement in the transport of isoflavones and their conjugates.

5. Enzyme-transporter interplay and enteric recycling

As already explained above, the isoflavone glycosides are enzymatically broken down into their aglycones in gut by bacterial enzymes, get transported into the enterocytes where they are either metabolised by conjugating enzymes or circulated to liver by the portal vein where again they are eventually converted into their phase II conjugates. In general, these Phase II conjugates, being negatively charged, are polar in nature and have poor membrane permeability. This necessitates carrier mediated transport of these conjugates for their biliary and intestinal excretion either basolaterally or apically. Isoflavone conjugates, as stated in the previous section, have been found to be substrates of ABC efflux transporters, BCRP and

MRP2, which are expressed apically. This leads to the efflux of isoflavone conjugates into intestinal lumen from enterocytes and via bile from liver. In the intestine, the Phase II metabolites are acted upon by the bacterial glucuronidases and sulfatases to release the aglycone which are reabsorbed in the colon and recirculated in a similar fashion [94, 95]. This enzyme-transporter coupling is an important phenomenon affecting the bioavailability of isoflavones[96]. In one of the initial studies carried out to explain this event, Jeong and group found that for formononetin, the rates of formation of glucuronide and sulfate was the rate limiting step in their disposition in Caco-2 cells while in mouse, the intestinal excretion of glucuronide was limited by their rate of excretion from enterocytes. However, for formononetin sulfate,efflux transporterslimited the excretion only in the upper intestinal segment [92]. Zhu *et al*. proposed that the intestinal disposition of genistein via sulfation pathway depended predominantly on a synchronized action of SULTs and BCRP. SULTs catalyse the biotransformation of genistein into its corresponding sulfate conjugates. This is a reversible process and, thus, the intracellular concentrations of genistein and genistein sulfate are important for deciding the course of this reaction. These concentrations, in turn, are controlled by BCRP since both genistein and genistein sulfate are substrates of BCRP. A co-ordinated interaction between SULTs and BCRP, hence, decidesthe formation and excretion of genistein sulfate [89]. The importance of thecontribution of Phase II enzymes and transporters is also explained in the study carried out by Jia and co-workers. They found that more formononetin metabolites were excreted in both intestine and liver than biochanin A metabolites when pure compounds of both isoflavones were studied in rat perfusion model and Caco-2 cells. However, on incubation with rat liver and intestinal microsomes, biochanin A was metabolised much faster than formononetin in both the cases. Since excretion of conjugates involves two processes, conjugate formation and their intestinal efflux, it proves that the rate of formation of metabolites alone cannot predict their excretion [97]. The relative participation of both must be considered in order to understand the exact disposition scheme of isoflavones.

This enzyme-transporter coupling leads to enterohepatic and enteric recycling. Enterohepatic recycling has been considered to play an important role in determining the bioavailability of a number of drugs such as oral contraceptives, digoxin *etc*. [98]. Enterohepatic recycling of genistein in rats has been studied by Sfakianos and group. They demonstrated that genistein infused either into duodenum, mid small intestine or portal vein was taken up by liver and excreted into bile as 7-O-β-glucuronide. Further they observed that the main site of glucuronidation was intestinal wall since the portal blood collected after the*in vivo* infusion of genistein into duodenum contained mostly 7-O-β-glucuronide [76]. For isoflavones, however, the transporter-mediated intestinal excretion of Phase II metabolites/enzyme-transporter coupling leads to another kind of recirculation known as enteric recycling. Enteric recycling involves the active transport of the Phase II conjugates formed in the enterocytes back into the intestinal lumen by efflux transporters, where they are deconjugated by the gut microflora and then recirculates likewise. It has been proposed that enteric recycling might be more important in deciding their disposition than enterohepatic recycling [76, 97, 99]. Chen *et al*., recently, showed that although liver possessed higher metabolising efficiency than intestine for genistein and daidzein (200-2000% higher intrinsic clearance and >2-fold lower Km in liver microsomes), still intestinal glucuronidation was more important for their excretion. The higher concentration of isoflavone reaching intestinal enzymes was cited as a possible reason for the same. The greater role of presystemic intestinal metabolism consequently leads to a much important role of the apically located ABC transporters in enterocytes and subsequent enteric recirculation [99]. Thus, enzyme-transporter interplay determines the relative contribution of enteric and enterohepatic recycling to the *in vivo*

disposition of isoflavonoids by controlling the amounts of metabolites excreted by intestine and liver. The knowledge of mutual interaction between isoflavones, drug metabolising enzymes and efflux transporters could provide important clues in elucidating the structure-metabolism and structure-activity relationship, thus, allowing selection of the right candidate as an adjunct therapy for increasing the therapeutic efficacies of drugs.

6. Enzyme induction/inhibition and its clinical significance

There are numerous reports in literature which states that these isoflavones are not only the substrates of drug metabolizing enzymes and influx/efflux transporters but also are potent inhibitors of thesame. Isoflavones have been reported as potent inhibitors of cytochrome P450 enzyme system particularly. The inhibitory potential of these isoflavones for various CYP isoforms and transporters may result in clinically serious herb-drug interactions which can sometimes be life-threatening.

Cytochrome P450 enzymes comprises of one of the most important drug metabolizing system in humans. Of the 30 CYP isozymes identified so far, broadly only 6 isozymes contribute determinantly to the metabolism of most drugs. These are CYP1A2, 3A4, 2C9, 2C19, 2D6 and 2E1. Around 50% of the drugs are metabolized by CYP3A4, the major isozyme of the CYP3A subfamily constituting 30% of the CYPs present in liver. CYP3A5 is the other important isoform of this subfamily. These are also the main isoforms present in intestine [100].Biochanin A and peurarin have beenshown to inhibit rat CYP3A activity [101, 102]. CYP1A2 is involved in the metabolic activation of procarcinogens to carcinogens and also in the metabolism of drugs like caffeine, theophylline, warfarin and desipramine [100]. Genistein anddaidzein were found to inhibit CYP1A2 activity [103, 104] whereas peurarin induced CYP1A2 activity in rats[101]. CYP1B1 metabolises procarcinogens and xenobiotics and has been detected in human tumors of breast, lung, colon, brain *etc*. It wasinhibited by genistein, daidzein, formononetin and biochanin A [105]. CYP2A6is the Phase I enzyme responsible for 7-hydroxylation of coumarin. It metabolises nicotine, alfatoxin B1 and coumarin type alkaloids. It was inhibited by daidzein, genistein and glycitein [106]. CYP2E1 involved in the metabolism of phenacetin and some of the low molecular weight toxins was inhibited by peurarin in rats at a dose of 100mg/kg[101]. Genistein and daizein have also been show to inhibit Phase II conjugating ezyme, UGT. However, human SULT1A1 and 2A1 were induced by genistein [107]. Table 2 gives a brief account of the metabolic enzymes mediated isoflavone-drug interactions.

Table 2: CYP, UGT and SULT mediated drug interactions involving isoflavones as modulating agents.

Interacting isoflavone	Precipitant drug	Test system	Observation	Mechanism implicated	Reference
Genistein,Daidzein	Oestradiol	Rat	Inhibition of sulfonation and beta-glucuronidation	Inhibition of SULT1A1 and UGT	[108]
	Dehydroisoandrosterone (DHEA)		Inhibition of sulfonation	Inhibition of SULT1B1	
	4-Methylumbelliferone(4-MU)		Inhibition of beta-glucuronidation	Inhibition of UGT	

Daidzein	Theophylline	Humans	AUC, C_{max} and $t_{1/2}$significantly increased	Inhibition of CYP1A2 activity	[103]
Daidzein	7-Ethoxyresofurin	Recombinant human CYP1B1	Decreased 7-ethoxyresorufin *O*-deethylation	Inhibition of CYP1B1	[105]
Genistein					
Formononetin, Biochanin A				Competitive substrates	
Genistein	Tamoxifen	Rat liver microsomes	Inhibition of α-hydroxylation	Inhibition of CYP1A2	[104]
Genistein,Daidzein,Glycitein	Nicotine	Recombinant human CYPs, Japanese human volunteers	Inhibition of nicotine-C-oxidation	Inhibition of CYP2A6	[106]
Biochanin A	Paclitaxel	Rat	AUC and C_{max}markedly increased	Inhibition of Pgp, CYP3A and OATP3 (preferential inhibition of OATP3 over Pgp)	[102]
	Digoxin		AUCand C_{max}increased		
	Fexofenadine		AUCandC_{max}decreased		
Genistein	Paclitaxel	Rat	AUC and C_{max} of orally administered paclitaxel increased significantly	---	[109]
Genistein	---	HepG2 cells, Caco-2 cells	Increased mRNA expression	Dose dependent induction of hSULT1A1 and hSULT2A	[107]

Besides, Genistein and daidzein have been reported to be inhibitors of non-Pgp mediated ABC transporters via MRP-1 and BCRP inhibition [110-116] while Biochanin A has been shown to inhibit P-gp activity in addition to MRP1 and BCRP [102, 111, 117-119]. Soy and red clover isoflavones have also been identified as inhibitors of organic anion transporter peptide-B (OATP-B) [102, 120, 121]. In a study conducted to undermine the effects of structural changes in isoflavones on their MRP1 inhibition potential, present in erythrocytes, Gania-Pietrzak *et al.* found that O-demethylation did not significantly alter their influence on MRP1 transporter activity. Genistein and biochanin A both showed concentration dependent inhibition of MRP1while daidzein and formononetin showed only weak modulatory effect [122].Table 3 gives a brief account of transporter mediated isoflavone-drug interactions.

Table **3**: Transporter mediated drug interactions involving isoflavones as modulating agents.

Interacting isoflavone	**Precipitant drug**	**Test system**	**Observation**	**Mechanism implicated**	**Reference**
Genistein	Daunorubicin	Non-Pgp MDR cell lines (GLC4/ADR, SW-1573/2R120, HT1080/DR4, MCF7/Mitox and HL60/ADR)	Increased accumulation	Non-P-gp mediated inhibition of multidrug resistance proteins	[112]
Genistein	29,79-bis-(carboxypropyl)-5(6)-carboxyfluorescein (BCPCF)	Human erythrocytes	Inhibition of BCPCF efflux	Inhibition of BCRP	[116]
Genistein	Daunorubicin (DNR)	Non-Pgp MDR overexpressing GLC4/ADR small cell lung cancer cell line	Apparent K_m value of DNR increased	Non-P-gp mediated inhibition of ABC transporters	[110]
Genistein, Biochanin A	Daunomycin,Vinblastine	Human pancreatic adenocarcinoma(Panc-1) cells	Significantly increased accumulation	Inhibition of MRP1-mediated drug transport	[111]
Biochanin A	Digoxin, Vinblastine	Caco-2 cells	Increased accumulation	Inhibition of P-gp-mediated efflux	[117]
Daidzein	Mitoxantrone, bodipy-FL-prazosin	BCRP overexpressing cell lines	Significantly increased accumulation	Inhibition of BCRP	[114]
Biochanin A, Genistein	[^{3}H] Dehydroepian-drosterone sulfate (DHEAS)	OATP1B1 expressing and negative HeLa cells	Inhibition of uptake	Inhibition of OATP1B1	[121]
Soyabean isoflavones	Estrone-3-sulfate	HEK293 cells	Inhibition of uptake	Inhibition of OATP-B mediated drug absorption	[120]
Biochanin A	Paclitaxel	Rat	AUCand C_{max}markedly increased	Inhibition of Pgp, CYP3A and OATP3	[102]

	Digoxin		AUCand C_{max}markedly increased	(preferential inhibition of OATP3 over P-gp)	
	Fexofenadine		AUCand C_{max}markedly decreased		
Genistein, Daidzein	Nitrofurantoin	Assaf sheep	Decreased levels of nitrofurantoin in milk	Inhibition of BCRP/ABCG2	[115]
Biochanin A	Daunomycin	P-gp-overexpressing MCF-7/ADR cells,	Increased accumulation	Inhibition of P-gp	[119]
Genistein, Daidzein	Nitrofurantoin	Wild-type and Bcrp1-/- mice	Increased plasma conc., decreased biliary excretion, decreased secretion in milk	Inhibition of Bcrp	[113]
Biochanin A	Mitoxantrone	MDCK/BCRP	Increased intracellular accumulation	Inhibition of Bcrp1-mediated efflux	[118]

The nature and extent of these interactions is important since isoflavones may constitute a major part of dietary supplements or daily diet in soy consuming regions and could lead to potential drug-drug or diet-drug interactions. Most of these interaction studies are performed *in vitro*. However, the indispensible need of *in vivo*data is justified by the lack of correlation found between the *in vitro* and *in vivo* findings. In a study undertaken to determine the effect of biochanin A on P-gp, Zhang *et al*. found that it had an inhibitory effect on P-gp causing increased accumulation of P-gp substrate, daunomycin, in P-gp overexpressing MCF-7/ADR cells. To support these results, they performed a similar study *in vivo* using doxorubicin, cyclosporine A, and paclitaxel as P-gp substrates. No significant change was observed in the pharmacokinetics of any of these susbstrates except for a moderate interaction in case of paclitaxel on oral or/and intraperitoneal administration of biochanin A [119]. The complexity of the situation and the discrepancy in these results may be explained by the fact that isoflavones may influence a number of metabolic enzymes and transporter carriers simultaneously making their *in vivo* disposition prediction from *in vitro* data a rather challenging task[123].Thus, it would seem a good option to investigate these interactions in animal models. However, there may be significant species differences in the type and degree of interactions observed during these studies as has been seen with other flavonoids [124, 125], depending on the presence/absence and the expression levels of proteins. The species differences could also stem from the differences in metabolism of isoflavones in different species. A comparison in the isoflavone metabolic phenotype in female Sprague-Dawley rats, Hampshire/Duroc Cross pigs, cynomolgus monkeys and women showed remarkable differences in soy metabolism. While equol was detected as a major circulating metabolite in the serum of rats and monkeys, it was negligibly found in the serum of either pigs or women. Aglycones were the main form of soy isoflavones excreted in rats (33-47%) and monkeys (90-97%) while in pigs and women, they were excreted as glucuronides (82-87%) and

sulfates (10-14%)[126].Zhu *et al.* observed that the rate of formation and excretion of genistein conjugates varied significantly in rats and mice. Only the glucuronide genistein conjugate was detected in rats perfusate while both glucuronide and sulfate conjugates were detected in mouse perfusate during intestinal perfusion study. Using S9 fractions, the rate of glucuronidated was two-fold higher in rats than mice while the rate of sulfation of genistein was 20-fold in mice than rats. Also, glucuronide intestinal excretion rate was significantly higher in mice[89]. Similar differences were reported for formononetin conjugates as well. Both formononetin glucuronide and sulfate were excreted in mouse intestinal perfusate while in rats, only glucuronide was excreted[92].Recently, Setchell *et al* showed that Phase II metabolism of genistein and daidzein markedly differed in humans and rodents.The capacity and extent of conjugation was found to be greater in humans than in either rats or mice. The plasma percentage of unconjugated genistein was 4.0 ± 0.6%, 4.6 ± 0.6%, 11.6 ± 0.9%, and 30.1 ± 4.3%in Sprague-Dawley rats and C57BL/6, nude, and transgenic AngptL4B6 mice strains, respectively, when fed soy-containing diets. These concentrations were around 20, 23, 58, and 150 times those in humans. Similarly, for daidzein, the percentage unconjugated in plasma at steady state was found to be 8.1± 1.1%, 7.4 ± 0.7%, 16.1 ± 1.2% and 32.7 ± 4.7% in Sprague-Dawley rats and C57BL/6, nude, and transgenic AngptL4B6 mice strains, respectively. These findings further lead to the important question of the clinical relevance of these rodent models for studying isoflavones[127]. This also provides a reason to expect that significantly varying results may be observed in different human populations owing to their pharmacogenetic and pharmagenomic differences. Further, the interaction could also depend on the type of substrate. For example, the transport of BCRP substrates, topotecan and albendazole sulphoxide (ABZSO) across BCRP over-expressing MDCKII cells was blocked by BCRP inhibitor Ko143. ABZSO transport was also inhibited by P-gp inhibitors ivermectin, LY335979, PSC833, and the P-gp/Bcrp inhibitor ritonavir, however, these inhibitors showed no effect on transport of topotecan. For this finding, Muenster *et al*. suggested that different modes of substrate and inhibitor binding to BCRP could cause the discrepancy in the result observed[128].Another important facet of co-consumption of many isoflavones is the effect of one isoflavone on pharmacokinetics of another isoflavone as was exemplified by the increased total AUC of biochanin A on coadministration with quercetin and (-)-epigallocatechin-3-gallate in case of flavonoids [129]. In a similar context, when isoflavones are taken in form of dietary supplements or as a part of diet, they are present in combination rather than singly, thus, their mutual interactions should also be considered.

Apart from inhibition/induction of Phase I and Phase II enzymes and transporter proteins, isoflavones have been shown to modulate the activity of other enzymes also.Vera *et al.*observed that genistein was a potent inhibitor of functional activity of mammalian facilitative hexose transporter GLUT1. The presence of genistein in assay medium inhibited the transport of dehydroascorbic acid, deoxyglucose and methylglucose across human myeloid HL-60 leukemia cells, GLUT1 over-expressing transfected chinese hamster ovary (CHO) cells and human erythrocytes with 50% inhibition observed at 12-15 μM. The inhibition was competitive (Ki=12 μm for deoxyglucose and methyglucose),dose-dependent but cell-independent and the same dose-effect curves were observed for all the three types of cells studied. This also indicated that the effect of genistein on GLUT1 did not involve its inhibition of tyrosine kinase phosphorylation since human erythrocytes do not express tyrosine kinase. However, no such effect on GLUT1 transporter was seen with daidzein[130].In a comparative study undertaken to elucidate the effect of various flavonoids on phosphodiesterase (PDE) enzyme, isoflavones were found to be inhibitors of this enzyme. Genistein non-selectively inhibited PDE1, 2, 3, 4 and 5 with IC_{50} of 16.8 ± 2.3, 1.7 ± 0.2, 12.9 ± 5.2, 9.5 ± 1.9, 73.9 ± 7.1μM. Daidzein and prunetin selectively inhibited PDE3 and

PDE4 with IC_{50} of 28.6 ± 8.5 and 61.9 ± 17.3 μM respectively. Biochanin A inhibited PDE1, 2 and 4 with IC_{50} of 29.1 ± 0.3, 27.9 ± 4.1 and 8.5 ± 0.1 μM [131]. Further studies have revealed that biochanin A could prove useful in treating asthma or chronic obstructive pulmonary disease (COPD) because of its selective inhibition of PDE4 at a lower IC_{50}. Ko *et al* showed thaton oral administration, it significantlyattenuated airway resistance (R_L), enhanced pause (P_{enh}), and increased lung dynamic compliance (C) values[132]. Soy isoflavones, genistein and daidzein, and their glycosides have been reported to be inhibitors of lipoxygenase enzyme (LOX). The addition of genistein or daidzein to soy LOX1-linoleic acid complex resulted in conversion of catalytically active ferric form of LOX to its resting ferrous form and consequently, the disappearance of the positive dichroic band at 425 nm, thus, proving the isoflavones to be redox inhibitors. The same results were obtained with genistin and daidzin suggesting that glycosylation at C-7 had no effect on theirredox inhibition potential. The inhibition was non-competitive and reversible [133].Chou *et al.*showed that genistein and daidzein non-competitively inhibited glucose, *N*-acetylglucosamine and sulphate transport in isolated rat liver lysosomes. Addition of 50μM genistein in the medium outside lysosomes resulted in inhibition of both glucose uptake and glucose efflux in a dose-dependent manner. Genistein also inhibited the activity of two other lysosomal transport systems, *N*-acetyl-D-glucosamine and sulphate anion transport, with similar profiles. Daidzein likewise inhibited the transport of glucose and *N*-acetylglucosamine, however, it was less potent than genistein and affected sulphate uptake across rat lysosomes only negligibly. The presence of both genistein and daidzein did not give additive results, suggesting that both work by same mechanism to cause this inhibitory effect. Since, daidzein did not inhibit tyrosine kinase, it implied that tyrosine kinase phosphorylation is not the mechanism underlying the effects of genistein on lysosomal transporters [134].The estrogen-sulfating cytosolic sulfotranferase enzyme,present in zebrafish namely SULT1 ST#2 was competitively and concentration dependently inhibited by genistein and daidzein with IC_{50} values of 2.5 and 8 μM, respectively. Addition of either genistein or daidzein to the sulfotranferase assay mixture consisting of recombinant zebrafish SULT1 ST#2 and examining its 17β-estradiol sulfating activity resulted in an increase in the K_m value of 17β-estradiol while not having any effect any pertinent effect on the V_{max} value of the reaction [135].

Isoflavones undergo extensive phase II metabolism as explained above. The conjugated metabolites of isoflavones have also been shown to be inhibitors of specific enzymes. The activity of sterol sulfatase, known to be involved in the conversion of estrone and dehydroepiandrosterone sulfate conjugates into their respective estrogenic steroids, was inhibited by sulfoconjugates of daidzein. Daidzein-4′-*O*-sulfate and daidzein-7,4′-di-*O*-sulfate competitively inhibited sterol sulfate with K_i values of 5.9 and 1 μM in hamster liver microsomal fraction using DHEA as substrate. In addition, these also inhibited hydroxysteroid and phenol sulfotransferases, however, with IC_{50}>100 μM for both daidzein-4′-*O*-sulfate and daidzein-7,4′-di-*O*-sulfate. [136].These inhibitory effects of isoflavones and their conjugates on enzymes other than CYPs, UGTs and SULTs could well form the basis of their clinical use. For example, the usefulness of biochanin A for treating COPD has been attributed to its inhibition of PDE andthe potential anti-tumor activity of daidzein sulfoconjugates due to their inhibition of sterol sulfate.

7. Conclusion

Isoflavones represent a special class of bioactive non-nutrients which have been associated with a myriad of preventive and curative health related effects, particularly involving their potential to prevent or treat hormonal-dependent disorders. Owing to their multifaceted therapeutic benefits, these dietary isoflavones have generated much interest. However, these

isoflavones show poor oral bioavailability hindering their development as potential drug molecules. Marked first pass metabolism as well as extensive conjugation with hydrophilic endogenous substrates such as glucuronic acid, sulfate *etc.* has been cited as the main reason responsible for their low oral exposure. The gut microflora also plays an important role in metabolism of isoflavones by initially deglycosylating the isoflavones into their aglycones, converting the aglycones into their metabolites and then contributing to the deconjugation of their phase II conjugates. This entero-hepatic as well as enteric recycling is considered to play a very important role in the biological disposition of isoflavones.The involvement of efflux transporters (BCRP, MRP2) further complicates their*in vivo* disposition and excretion.
Dietary supplements rich in isoflavone content are increasingly been prescribed and consumed particularly in the western countries. There is a common belief that consumption of these supplements is safe since these are obtained from natural sources. However, this is a dangerous oversimplication. Many clinically serious drug-herb interactions have been reported in the literature due to alteration of pharmacokinetics of co-administered drugs, affecting their safety and efficacy, in the presence of these isoflavone supplements. This is because, on one hand the isoflavones act as substrate for a number of enzyme and transporter systems, while, on the other, they act as potent inhibitors of the same. They modulate the activity of CYPs, UGTs, SULTs, BCRP, MRP1, P-gp and OATP. Thus, if these dietary molecules are to be developed as future drugs, due consideration should be given to their full pharmacokinetic characterization as well as drug interaction inducing potential.

Conflict of interest

The authors declare that they have no conflict of interest.

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