EXPERT OPINION

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Amorfrutins are efficient modulators of peroxisome proliferator-activated receptor gamma (PPARγ) with potent antidiabetic and anticancer properties: a patent evaluation of WO2014177593 A1

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Introduction: PPARγ is an essential regulator of lipid, glucose, and insulin metabolism. PPARγ full agonists, such as thiazolidinediones, are the mainstay drugs for the treatment of type 2 diabetes; however, undesirable clinical side effects have contributed to poor compliance with therapy and limited their full therapeutic potential. In the last few years, many efforts have been made in the discovery and development of selective PPARγ modulators (SPPARγMs) as safer alternatives to PPARγ full agonists.
Areas covered: This application claims the plant-derived amorfrutins or their synthetic analogs as SPPARγMs with potential to exhibit glucose-lowering effects without provoking side effects associated with full PPARγ activation. Specifically, the *in vivo* glucose-lowering properties of the high-affinity SPPARγM amorfrutin B are described. Moreover, examples of this class of compounds exhibit interesting antiproliferative activities.
Expert opinion: The patent (WO2014177593 A1) under discussion proposes enriching functional food products or phytomedical extracts with safe licorice extracts, containing sufficient amounts of amorfrutins, with the ultimate goal of inhibiting the early development of disorders such as insulin resistance. Interestingly, some example compounds show anticancer properties in colon, prostate, and breast malignancies. However, further *in vivo* investigations of the claimed compounds for these specific indications will be necessary to definitively support their clinical applications.

Keywords: anticancer, diabetes, insulin resistance, natural product, peroxisome proliferator-activated receptor, selective PPARγ modulators

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1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the II class of nuclear receptors superfamily. They are ligand-dependent transcriptional factors involved in the control and expression of several genes implicated in glucidic and lipidic homeostasis and energetic balance. PPARs are lipid sensors activated by specific natural and synthetic ligands, which play an important role during cell signaling [1]. PPARs dynamically shuttle between nucleus and cytoplasm, although they constitutively and predominantly appear in nucleus [2]. The nuclear-cytoplasmic shuttling of

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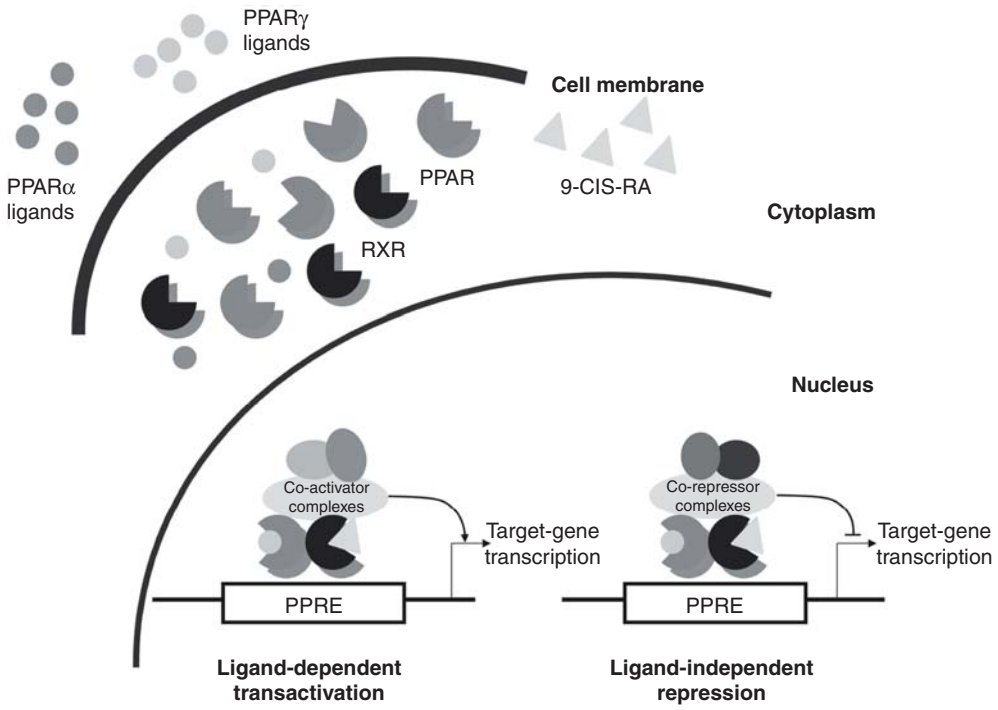

Figure 1. Transcriptional Regulation by PPARs. After ligand binding, PPARs undergo conformational changes, which lead to recruitment of RXR and coactivators. The resultant heterodimers bind to specific DNA response elements called PPREs, causing target gene transcription (ligand-dependent transactivation). In the absence of ligands, PPARs bind the promoters of their target genes and repress transcription by recruiting the co-repressor complex (ligand-independent transrepression).

PPARs is regulated by respective PPAR ligands [2]. In unstimulated cells, PPARs are located in the cytoplasm as heterodimers complexed to their repressors. After ligation with their agonists, PPARs heterodimerize with retinoid X receptor (RXR) and co-activators such as p300 are recruited [3,4]; this complex is translocated to the nucleus where it recognizes specific DNA sequence elements termed as peroxisome proliferator response element (PPRE) in promoters of target genes (**Figure 1**). The transcriptional activity of PPARs is finely regulated by co-activators or co-repressors, which modulate signaling and interaction with the basal transcription machinery [4]. In the absence of ligands (ligand-independent repression), the PPAR/RXR complex is bound to transcriptional co-repressors and histone deacetylases, which prevents its binding to PPRE [5]. Upon ligand activation (ligand-dependent transactivation), PPARs undergo conformational change, and recruit co-activators such as p300/CBP and p160 to displace co-repressors, resulting in binding of target gene PPRE and inducing transcription.

To date, three human PPAR isoforms have been identified: PPARα, PPARβ/δ (also known as PPARβ or PPARδ), and PPARγ. Each isoform has a distinct tissue distribution, different activating ligands, and selective function in the regulation of metabolic processes in the organism (**Table 1**) [6-8].

PPARγ is the target of thiazolidinediones (TZD) class of insulin-sensitizing drugs, clinically employed in patients with type 2 diabetes [9]. However, these PPARγ activators have undesirable clinical side effects such as weight gain, peripheral edema, bone loss, increased risk of congestive heart failure, and other disorders that contribute to a poor compliance with therapy in diabetic patients.

Selective PPARγ modulators (SPPARγMs) are novel PPARγ ligands that can promote a differential cofactor recruitment profile, leading to specific patterns of gene expression. In preclinical species, SPPARγMs were shown to retain antidiabetic efficacy comparable to that of full PPARγ agonists while displaying reduced PPARγ mechanism-based adverse effects [10]. Thus, the use of SPPARγMs is a promising approach for developing glucose-lowering agents with an acceptable safety profile [11].

The patent application [12], from Max-Planck Institute and Boyce Thompson Institute For Plant Research, that is the subject of this evaluation, specifically claims that the amorfrutins, a family of phenyl terpenoid natural products isolated from the edible parts of the legumes *Glycyrrhiza foetida* and *Amorpha fruticosa*, selectively modulate PPARγ activity at low nanomolar concentrations, offering a promising approach to prevent or treat complex metabolic diseases such as insulin resistance or type 2 diabetes.

Table 1. Ligands, tissue distribution, and main biological effects of PPARs.

Receptor	Ligand	Tissue distribution	Function
PPARα	FA, fibrates, Eicosanoids	Heart, liver, kidney, adipose tissue, skeletal muscle	FA catabolism, lipid homeostasis, anti-inflammatory
PPARγ	PUFAs, 15d-PGJ2, TZD	Adipose tissue, macrophages, kidney pancreas, spleen colon and large intestine, heart, skeletal muscle	Glucose homeostasis, insulin homeostasis, adipocyte differentiation, macrophage function, anti-proliferating, anti-angiogenic
PPARβ/δ	FA, L-165041, GW0742, GW501516	Ubiquitous, highly expressed in skin, brain and adipose tissue	FA catabolism, glucose homeostasis, adipocyte differentiation, anti-inflammatory, carcinogenesis (?)

15d-PGJ2: 15-deoxy-delta-12,14-prostaglandin J2; FA: Fatty acids; PUFAs: Polyunsaturated FA; TZD: Thiazolidinediones.

2. Chemistry

Amorfrutins and their synthetic analogs claimed in this patent were isolated from 20 microbial strains (of terrestrial and marine origin) and different plants or obtained by chemical synthesis. Compound isolation was carried out by extracting twice dried seeds, roots, aerial parts of plants or fungal strains with methanol/methyl-*tert*-butyl ether (MTBE) or methanol/acetone. By repeated chromatography (stationary phase RP-8 and RP-18, mobile phase methanol-water, and acetonitrile-water), natural products were isolated in a purity of > 70%. Structures were elucidated by interpretation of LCMS, 1D and 2D NMR (HSQC, HMBC, HH-COSY) data.

The 43 compounds described in the document are structurally simplified by the benzene ring (**I**) (**Figure 2**), wherein R_1–R_6 represent simple or more complex aliphatic or aromatics groups described in detail in the patent application. A variant to the core scaffold benzene ring is that R_2 together with R_3 or R_3 together with R_4 form with the two carbons of the benzene ring to which they are attached 2,3-dihydrofuran or furane rings (**II**) substituted with saturated or unsaturated, linear or branched R_7 alkyl groups (**Figure 2**).

The structures of the most interesting novel amorfrutins disclosed are reported in **Figure 2**. Amorfrutins of general formula **10**,**11**,**12** disclosed in the invention can be efficiently synthesized by three steps starting from the ketone **3** and the malonate derivative **4** (**Figure 3**).

To access ketone **3**, hydrazone **5**, obtained from the corresponding methyl ketone, is reacted with commercially available brominated derivative **6** in the presence of a strong base such as lithium diisopropylamide in anhydrous THF. Examples of suitable brominated derivative **6** are 1-bromo-3-methylbut-2-ene and (2*Z*)-1-bromo-3,7-dimethylocta-2,6-diene.

After treatment of diethyl malonate **8** with Mg turnings in absolute ethanol and a catalytic amount of CCl_4, the intermediate magnesium salt is reacted with acyl chloride **7** to provide enol **9**. Suitable acyl chlorides **7** are hydrocinnamoyl chloride, pentanoyl chloride, butanoyl chloride, propanoyl chloride, ethanoyl chloride, and formyl chloride. Enol **9** is converted to corresponding chloride **4** by treatment with phosphoryl chloride ($POCl_3$) and triethylamine (Et_3N).

Subsequent treatment of chloride **4** with ketone **3** provides amorfrutin analog **10** that can be further methylated to provide the analog **11**. Cleavage of the methyl ester on methylated analog **11** furnishes amorfrutin **12**.

3. Biology and action

The compounds described in this patent were evaluated *in vitro* by binding and cofactor recruitment assays and by transcriptional activation assays in primary human adipocytes and murine preosteoblasts, as well as *in vivo* using insulin-resistant high-fat-diet-fed C57BL/6 mice. **Table 2** summarizes the binding and activation of PPARs by the most interesting amorfrutins disclosed in the patent.

Among the various compounds tested, amorfrutin B emerged as the most selective and potent SPPARγM. Amorfrutin B had the lowest, nanomolar, binding affinity constant (K_i) to purified PPARγ (K_i = 0.02 μM; **Table 2**) similar to the standard PPARγ-targeting drug rosiglitazone (RGZ) (K_i = 0.007 μM), and 12 times lower than amorfrutin 1 (K_i = 0.24 μM). This compound activated chimeric Gal4-PPARγ-dependent reporter gene expression as partial agonist (with EC_{50} = 0.07 μM and maximal efficacy 4-fold lower than RGZ). Amorfrutins 1 – 4 also were able to bind to purified PPARγ (**Table 2**) and to activate chimeric Gal4-PPARγ-dependent reporter gene expression as partial agonists, although less potently than amorfrutin B. In addition, both amorfrutins 1 – 4 and amorfrutin B bound to the subtypes PPARα (which are mainly expressed in the liver) and PPARβ/δ (which are ubiquitously expressed), with amorfrutin B exhibiting lower micromolar K_i values (K_i = 2.6 and 1.8 μM for PPARα and PPARβ/δ, respectively).

The inventors of this patent also investigated the potential transcriptional activation of these compounds using reporter gene assays. All compounds showed PPARγ activation, with EC_{50} values ranging from 25 nM to 6.9 μM and transactivation efficacies ranging from 5 to 79% relative to RGZ. Once more, amorfrutin B diplayed nanomolar effective concentrations (EC_{50} = 0.07 μM) and reduced maximal PPARγ

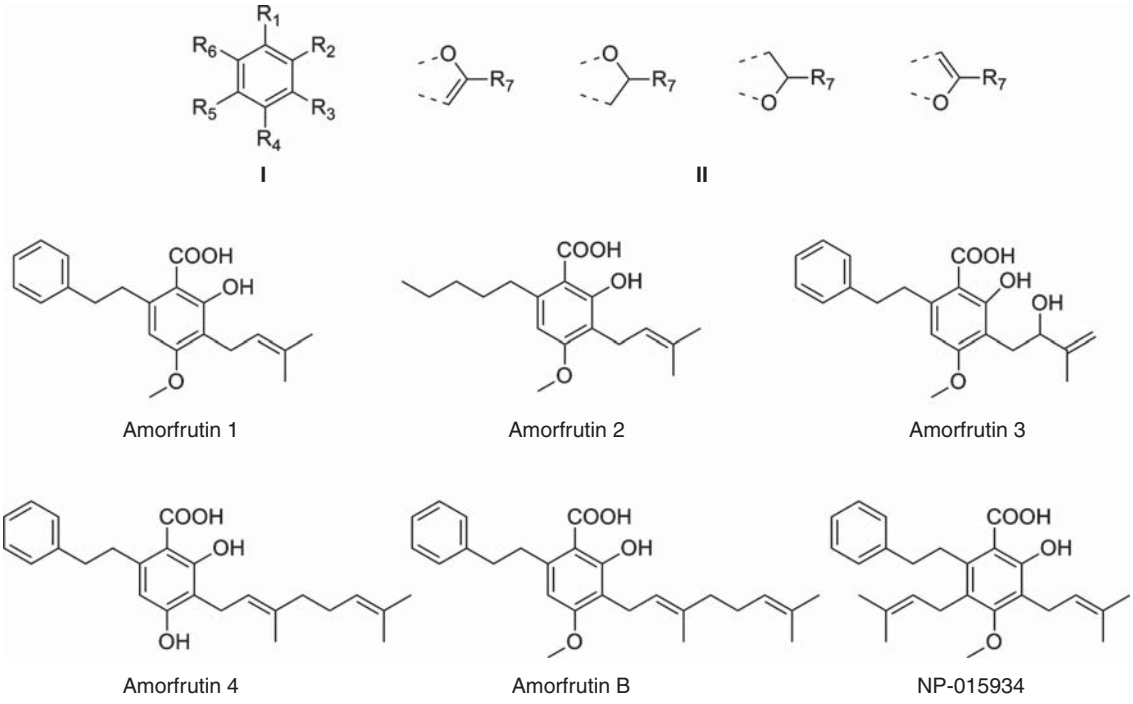

Figure 2. Structures of the most interesting amorfrutins described in the patent application.

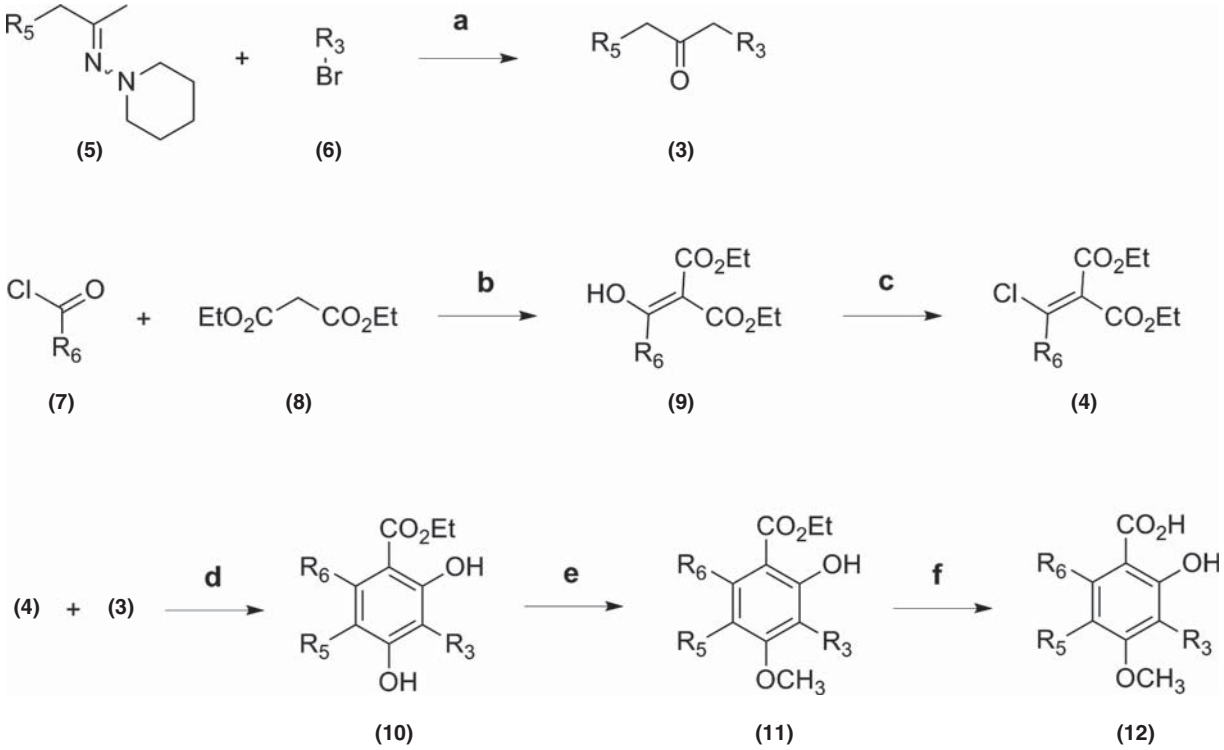

Figure 3. General synthetic route utilized to prepare the amorfrutins with general formula (10), (11), and (12) disclosed in this patent. (**a**) LDA, THF. (**b**) Mg turnings, EtOH, CCl_4. (**c**) $POCl_3$, Et_3N. (**d**) LDA, THF. (**e**) Iodomethane (MeI), $NaHCO_3$. (**f**) KOH, DMSO.

Table 2. Binding, activation, and cofactor recruitment of PPARs by amorfrutins.

Receptor	Amorfrutin 1	Amorfrutin 2	Amorfrutin 3	Amorfrutin 4	Amorfrutin B	RGZ
PPARα						
K_i (μM)	27	25	115	8.0	2.6	n.d.
EC_{50} (μM)	0.70	0.39	0.81	0.66	0.91	n.d.
Efficacy (%)	23	18	13	14	61	n.d.
PPARβ/δ						
K_i (μM)	27	17	68	6.0	1.8	n.d.
EC_{50} (μM)					0.74	n.d.
Efficacy (%)					3	n.d.
PPARγ						
K_i (μM)	0.24	0.29	0.35	0.28	0.02	0.007
EC_{50} (μM)	0.46	1.20	4.50	0.98	0.07	0.004
Efficacy (%)	39	30	22	15	25	100
NCoR						
EC_{50}/IC_{50} (nM)	51	32	2.8	34	60	23
Efficacy (%)	75	85	92	78	61	100

activation (efficacy = 25%). In contrast to the full PPARγ agonist RGZ, amorfrutins only partially induced transcriptional activation of PPARγ, suggesting that the compounds of this invention represent a new chemical class of SPPARγMs. Transcriptional activation of the PPARα subtype was additionally tested in the same reporter gene assay, with amorfrutin B exhibiting EC_{50} and efficacy values of 0.91 μM and 61%, respectively. Amorfrutins 1 – 4 showed EC_{50} and efficacy values ranging from 0.39 to 0.81 μM and 13 to 23%, respectively. Finally, amorfrutin B provoked the activation of the PPARβ/δ subtype at nanomolar concentrations (EC_{50} = 0.74 μM), although the efficacy of this activation was only 3%.

Interestingly, amorfrutin B induced partial recruitment of several PPARγ transcriptional coactivators and reduced binding of the corepressor nuclear receptor 1 with an IC_{50} value similar to RGZ (60 vs 23 nM with RGZ), but with lower maximal dissociation efficacy (61 vs 100% RGZ, **Table 2**).

Using a PPARγ activation assay, it was shown that amorfrutin B regulated gene expression in human adipocytes in a PPARγ-dependent manner, thus suggesting potential glucose-lowering effects with, presumably, lower susceptibility to adipose-derived body weight gain *in vivo* upon amorfrutin B treatment. Previous work has shown that phosphorylation of PPARγ at Ser273 by cyclin-dependent kinase 5 stimulates diabetogenic gene expression in adipose tissues [13]. Inhibition of this modification is a key therapeutic mechanism for antidiabetic drugs that bind PPARγ, such as TZDs and PPARγ partial agonists or non-agonists [13]. The inventors showed that amorfrutin 1 blocked the phosphorylation of PPARγ in visceral white adipose tissue of DIO mice [14], whereas amorfrutin B did not produce any significant change at this phosphorylation site [15]. In insulin-resistant, diet-induced obese and in genetic diabetes mouse models, treatment with 100 mg/kg/d of amorfrutin B strongly increased insulin sensitivity and glucose tolerance and improved many other physiological parameters [14,15]. Strikingly, early intervention with amorfrutin B inhibited the development of fatty liver during high-fat diet treatment of mice, an exciting feature to prevent metabolic liver diseases. In various diabetic mouse models, amorfrutin B did not show the unwanted side effects of the TZDs, such as weight gain or adverse effects on osteoblastogenesis and fluid retention.

Further, some amorfrutins disclosed in the patent application revealed concentration-dependent antiproliferative effects against HT-29 colon, PC3 prostate, and MCF-7 breast cancer cells with IC_{50} values ranging from 8.1 to 57.3 μM and with efficacies of up to 100% cancer cell death induction. One of the example compounds (NP-015934) was co-administered with cisplatin or irinotecan, showing additive effects on inhibiting HT-29 cell proliferation. The inventors suggested the involvement of apoptosis as a mechanism of compound-induced cancer cell owing to considerable caspase activation in all tested cancer cells, striking DNA fragmentation, substantial phosphatidylserine externalization, significant formation of reactive oxygen species, and loss of mitochondrial transmembrane potential.

4. Expert opinion

Given worldwide increases in the incidence of obesity and type 2 diabetes, new strategies for preventing and treating metabolic diseases are needed. Therefore, it is not surprising that this field attracts increasing attention of academic and pharmaceutical research to investigate potential exploitation as drug targets and/or new types of drugs.

The present patent represents a logical evolution of the work previously realized by the same inventors [14,15]. The compounds disclosed represent a family of structurally new and powerful natural antidiabetics, the amorfrutins, which are high-affinity SPPARγMs with potential to exhibit strong glucose-lowering properties without provoking side

effects associated with full PPARγ activation. In addition to amorfrutins, many other natural products have been found to act as SPPARγMs, partial, or dual agonists [16]. In particular, honokiol, magnolol, resveratrol and amorphastilbol have shown the same efficacy of amorfrutins in improving blood glucose levels and other relevant parameters in diabetic animals with few side effects compared with TZD treatments. Additionally, preclinical and Phase I/II clinical studies with synthetic SPPARγMs, INT131 [17], MBX-102 [18], MB-2044 [19], balaglitazone [20], and indeglitazar [21] have provided evidence of comparable or better insulin sensitization with respect to full agonist without PPARγ-mediated side effects. An analysis of published documents by Espacenet revealed that SPPARγMs as safer alternatives to PPARγ full agonists seem to represent yet a middlingly competitive field, which is so far mainly investigated by academia or industry with at least 78 published documents.

The invention claimed in this patent may be directed to enrich functional food products or phytomedical extracts with safe licorice extracts, containing sufficient amounts of amorfrutins, with the ultimate goal of inhibiting the early development of disorders such as insulin resistance. The central core of the compounds described in this patent is identical to that previously disclosed by these inventors [14,15], whereas a number of modifications are proposed to external "appendages". In addition, *in vitro* and *in vivo* glucose-lowering activities of only one example molecule, amorfrutin B, are described, although the synthetic scheme utilized to reach these compounds is well characterized and amenable to a more thorough exploration of the structure–activity relationships for this class of compounds. Pharmacokinetic studies in C57BL/6 mice demonstrated good absorption of amorfrutin B after oral dosing of 100 mg/kg (C_{max} = 30.4 mg/l; C_{min} = 0.015 mg/l; AUC = 85.4 mg/l × h) and a relatively long half-life ($t_{1/2}$ = 2.3 h; k_e = 0.307 h^{-1}; CL = 30.0 ml/h). Thus, the animal studies discussed, although quite promising and suggestive of an antidiabetic effect of amorfrutin B, need to be expanded to primates and then humans before more definitive conclusions can be drawn. Moreover, the authors did not report the *in vivo* experiments for the remaining example molecules, nor did the authors perform extensive carcinogenicity tests (2 y/two species), a Food and Drug Administration requirement for long-term studies in humans. Hence, although amorfrutins appear promising, it is impossible at present to predict whether they will have a better safety profile in humans than TZDs.

The novelty of the patent resides in the additional antiproliferative and pro-apoptotic properties of PPARγ-binding natural amorfrutins. Eight example molecules were demonstrated to suppress the growth of HT-29, PC3, and MCF-7 cancer cells, by inducing caspase-associated apoptosis via the mitochondrial pathway. However, the therapeutic claims for the treatment of many types of cancer are not supported by direct studies on the example compounds. Ample evidence in the literature supports PPARγ involvement in modulation of proliferation and apoptosis of many cancer cell types and its expression in many human tumors including lung, breast, colon, prostate, and bladder cancer [22-24]; however, the exact mechanisms underlying these effects are still being explored. In particular, it remains to be further explored whether activation of PPARγ itself or PPARγ-independent effects of amorfrutins contribute to the inhibition of cancer cell growth. Thus, further *in vitro* and *in vivo* investigations of the claimed compounds for these specific indications will be necessary to definitively support their clinical applications.

Declaration of interest

This work was supported by the Ministero dell'Istruzione, Università e Ricerca (MIUR-PRIN 2010–2011, prot. 2010W7YRLZ_003). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in/or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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