The mechanisms of Fenretinide-mediated anti-cancer activity and prevention of obesity and type-2 diabetes.

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ABSTRACT

Fenretinide remains the most investigated retinoid compound for the prevention of cancer. Its clinical use remains a genuine possibility due to a favourable toxicological profile and accumulation in fatty tissues. Like other well-characterised pharmacological therapies, Fenretinide has been shown to affect multiple signalling pathways. Recent findings have discovered additional beneficial properties the synthetic retinoid was not intentionally designed for, including the prevention of high-fat diet-induced obesity and insulin resistance. These preclinical findings in rodents are timely since obesity has reached pandemic proportions and safe effective therapeutics are severely lacking. Recent investigations have proposed various mechanisms of action for the beneficial effects of Fenretinide. This review covers the current knowledge about Fenretinide's use as a therapy for cancer and potential to treat obesity, insulin resistance and glucose intolerance. An overview of the signalling pathways manipulated by Fenretinide including retinoid homeostasis, reactive oxygen species generation and inhibition of ceramide synthesis will be presented and insights into apoptosis and/or autophagy induction by Fenretinide will also be discussed. The largely unexplored area of Fenretinide metabolites as alternative therapeutic options and how these may be relevant will also be presented. Fenretinide shows great promise but unfortunately evidence is lacking from clinical trials on Fenretinide's effectiveness in humans. Finally we identify what action can be taken to further progress the investigation of this extremely important retinoid.

1. Introduction.

Obesity, the condition of being overweight or to carry excess body fat is ubiquitous in today's society and is often stated as having reached epidemic status. Systematic analysis of health examination surveys and epidemiological studies has estimated that worldwide, more than 1.46 billion adults were overweight with a body mass index (BMI) of $\geq$25 kg/m^2 in the year 2008 [1]. Moreover, around a third of these individuals were classed as obese with a BMI of $\geq$30 kg/m^2 [1]. Of greater concern, obesity poses additional serious detrimental health consequences associated with perturbations to metabolic homeostasis. These include, but are not limited to, chronic diseases such as type II diabetes [2], cardiovascular disease [3] and the development of certain types of cancers [4]. Moreover, despite extensive pre-clinical and clinical research into these complex diseases, they continue to be the major causes of death worldwide. It is therefore imperative that efforts are made to reduce the levels of obesity and obesity-associated metabolic disturbances that are currently observed today in both the developed and developing world.

Unfortunately, education through the promotion of healthy lifestyles along with well-balanced diets appears to have had little impact on reversing the ever-expanding numbers of overweight and obese individuals. Thus, like the approach to target cancer with pharmaceutical therapy and/or prevention, an alternative approach to combat levels of obesity would be through the development of safe and effective pharmacological treatments. Despite the scale of the present situation, unfortunately very few therapeutic options are available [5]. More drugs are approved for the treatment of type II diabetes, however potentially dangerous side-effects are still encountered with their use [2,6]. Promisingly, vitamin A and its derivatives known as retinoids have been evaluated and used for the treatment of some types

of cancer and more recently, preclinical studies have suggested they may be useful for the prevention and/or treatment of obesity and type II diabetes.

2. Retinol metabolism and all-*trans*-retinoic acid signalling.

Vitamin A (or retinol) is the parent compound of all bioactive retinoids and is convertible to other natural forms through the retinol metabolism pathway. Active metabolites of retinol, primarily all-*trans*-retinoic acid (RA), act as important signalling molecules with the ability to induce gene expression through specific nuclear hormone receptors [7]. RA-receptor (RAR)s form heterodimers with retinoid-X receptors (RXR)s and bind to RA-response elements (RARE)s present in the promoters of target genes via the DNA-binding domain present within each receptor. As a result, the metabolism of vitamin A has been shown to play essential roles in the preservation of immune function, continued promotion of good vision and the development, growth and maintenance of multiple body tissues. Acquiring and maintaining a sufficient quantity of this fat soluble vitamin is therefore essential for life. Animals however do not have the capability to generate vitamin A via *de novo* synthesis. Vitamin A must therefore be obtained from dietary sources, stored in the liver and mobilised as required.

Dietary intake of vitamin A can be achieved through the absorption of pigments known as carotenoids from fruits and vegetables. These pro-vitamins can then be enzymatically cleaved and converted to compounds with the biological activity of retinol [7]. Alternatively, intake can be achieved by consuming animal tissue such as liver, where pro-vitamin A carotenoids have already been processed and stored in the form of retinyl esters. Although vitamin A is essential, excessive intake can be equally detrimental to life. Hypervitaminosis A can lead to toxicity of the liver, decreased bone mineral density and induce teratogenic effects in the developing embryo [7]. Additional concerns arise with the

use of retinoid therapy in women of child bearing age, as these compounds have the capability of inducing teratogenic effects in the developing conceptus. Vitamin A is a lipophilic, fat soluble molecule and therefore requires specific binding proteins in order to be transported in the circulation and within the cell. Despite this necessity, retinoid compounds are soluble in aqueous solutions at relatively low concentrations. For example, RA is water soluble up to concentrations of 210 nM at room temperature and pH 7.3 [7]. This makes retinoid compounds ideal morphogens. The generation of morphogen concentration gradients through diffusion allows for selective cellular differentiation to occur and determine tissue pattern during development [8]. As a result, the administration of retinoid compounds has been shown to provoke teratogenic effects in both animal models and humans. It has been suggested that chemical modification of the terminal-polar group of the retinoid molecule would offer a useful way to reduce toxicity but also modify activity, metabolism and tissue distribution of this class of compounds [9,10].

3. N-(4-hydroxyphenyl)retinamide; a synthetic retinoid.

3.1. Structural and advantageous properties of N-(4-hydroxyphenyl)retinamide.

N-(4-hydroxyphenyl)retinamide, otherwise known as 4-HPR or Fenretinide (FEN, used hereafter), is one such synthetic retinoid that was first synthesised in 1960s by R.W. Johnson Pharmaceuticals now part of Johnson and Johnson [11]. FEN shares a similar chemical structure with RA however it contains an amide linked 4-hydroxyphenyl group, which replaces the carboxyl polar end group of RA (Fig.1.). It is the addition of this bulky 4-hydroxyphenyl group which is thought to be responsible for a number of beneficial properties associated with FEN treatment, compared to alternative retinoid compounds such as RA

Since naturally derived vitamin A compounds such as RA and retinyl-acetate supplemented in large doses show liver toxicity with prolonged exposure this restricts their

potential use as medicinal agents. FEN on the other hand displays a decreased toxicological profile, which may occur due to a number of reasons. Chronic retinyl-acetate treatment results in the deposition of retinyl esters in the liver and subsequently causes hepatic toxicity. In contrast, FEN does not appear to be stored in the liver of rats [11]. This may be due to the observation that FEN and its metabolites are preferentially stored in fatty tissues such as mammary gland, which has been observed in both animal models and human studies [11,12]. Therefore, this characteristic appears to prevent FEN treatment leading to hepatotoxic accumulation and is highly advantageous compared to the use of natural forms of vitamin A as a therapeutic option. The specific accumulation of FEN in fatty tissues is also an beneficial property for the prevention/treatment of breast cancer, obesity and type II diabetes [11-14].

Encouragingly, studies performed in rats and rabbits have revealed that when FEN was given orally at 20 mg/kg/day, no adverse effects were observed in either species. At higher doses of 125-800 mg/kg/day, FEN was deemed to be only weakly teratogenic in these species [15]. Studies in hamsters dosed with up to 130 mg/kg of 13-*cis*-N-(4-hydroxyphenyl)retinamide also failed to induce a teratogenic response [10]. Genotoxic studies (the Ames mutagenicity test, a mouse lymphoma assay and a rat bone marrow cytogenetic assay) with FEN treatment reported all negative findings [16]. Together these results indicated that FEN is unable to induce point mutations or chromosomal aberrations and is therefore not a genotoxic compound.

3.2 Cancer chemoprevention trials

These desirable properties make the use of FEN as a therapeutic agent a genuine possibility. In agreement with this, due to the beneficial chemopreventive potential that FEN treatment has displayed during its early investigation in pre-clinical animal models [11], human clinical trials, predominantly for breast cancer chemoprevention, have demonstrated

that FEN is well-tolerated and compatible with long term treatment schedules [17]. In a large randomized trial of FEN to prevent second breast malignancy in almost 3000 women with early breast cancer, overall, FEN treatment for 5 years appears to have no statistically significant effect on the incidence of second breast malignancies of women with breast cancer [17]. A possible benefit was detected in premenopausal women, results that persisted in a 15-year follow-up [17,18]. These effects are potentially through an associated lowering of circulating IGF-1 levels, a potent stimulator of cell growth [17]. Combination therapy with low dose tamoxifen also did not reduce breast cancer events compared to placebo or single agents alone [19]. Unfortunately, overall these trials have yielded only preliminary data and new untested hypotheses.

FEN has also been widely studied in rodent bladder carcinogenesis models, where it has shown the highest therapeutic index among the retinoids tested, however in phase III trials it did not reduce the recurrence of bladder cancer in patients [20]. The lack of FEN efficacy in these trials has been suggested to be due to the dose used and subsequent tissue levels achieved, that was essentially too low to induce apoptosis, the major hypothesized mechanism of anti-cancer activity in cultured cells. FEN induces apoptosis in cells that are resistant to RA, suggesting that FEN-induced apoptosis may involve RAR–independent mechanism(s), such as increased generation of reactive oxygen species (ROS) and ceramide species and activation of stress kinases, endoplasmic reticulum (ER) stress and autophagy pathways [21]. However, high concentrations of FEN are required to induce apoptosis [22,23]. With this in mind, high doses of FEN and formulation within novel lipid matrices to improve FEN bioavailability and attain higher plasma concentrations have been tested in adults and in children with neuroblastoma with minimal toxicity [24,25]. Since higher plasma levels of FEN were achieved using this strategy, a phase II trial would now be recommended to further evaluate its anti-cancer activity.

4. Mechanisms of FEN-mediated anti-cancer activity.

Apoptosis is a physiological process of programmed cell death, is disrupted in various cancers and thus has been exploited as strategy to combat the disease, traditionally by inducing DNA damage with chemo- and radio-therapy. With an increased understanding of the intrinsic and extrinsic pathways of apoptosis (essentially mitochondrial mechanism and TRAIL-Fas-death receptor mechanism respectively) in recent years, novel approaches of targeting apoptosis have been tested in pre-clinical models and early phase clinical trials [26]. Natural retinoids like RA induce differentiation and/or cytostasis in target cells, while FEN can trigger apoptosis (at least in cultured cancer cells) via distinct biological effects.

4.1 Involvement of the canonical retinoid signalling pathway.

As a synthetic derivative of RA, it would be anticipated that similar to its natural counterpart, FEN would be an agonist for RARs and activate the retinoid signalling pathway. This however has proved to be a controversial issue. It was shown in one study that unlike RA, FEN bound very poorly to all three RAR isoforms [27], which may be due to the fact that FEN does not contain a carboxyl functional group (see Figure 1). In keeping with this interpretation, earlier investigations observed that FEN treatment was able to induce apoptosis in malignant hemopoietic cell lines, including those that were resistant to the effects of RA [28], implying a RAR-independent mechanism of action. Although FEN may also have RAR-independent mechanisms of action, some of which shall be discussed shortly, a number of studies have been conducted which established that FEN can bind to RARs and activate RAREs. It was found that FEN did display binding affinity with RARs, however only at 15% to that observed with RA treatment [29]. This finding implied that FEN could operate in a RAR dependant manner, however appeared to be less potent that RA. Additional reports have

confirmed this finding, since FEN can induce transcription of RARs as demonstrated by activation RARE reporter gene assays [23,30,31].

FEN has the ability to bind to serum retinol binding protein (RBP4, discussed later in section 6) however, no binding affinity has been observed between FEN and the cellular retinol or cellular RA binding proteins (CRBP or CRABP) [29]. Consistent with transactivation assays that indicate FEN can induce transcription via RARs [23,30,31], FEN was found to activate the RARE in the promoter for *Crbp1* and was actually observed to be a stronger activator than RA when in the presence of RXRγ-RARγ or RXRβ-RARγ heterodimers, which bind direct repeat (DR)-2 RAREs [31].

More recently, Y-J.Y Wan and co-workers have shown that FEN-induced apoptosis in FEN-sensitive Huh7 liver carcinoma cells involves a RARβ-dependant interaction with nuclear orphan receptor Nur77 that leads to nuclear export of the two proteins [32]. Nur77 has been reported to relocate to mitochondria where it participates in the conversion of Bcl-2 into a pro-apoptotic molecule [33]. In contrast, a subsequent transcriptome analysis in Huh7 cells identified that FEN (unlike RA) specifically induced TRAIL-Fas-death receptor mediated apoptosis by increasing the expression of pro-apoptotic genes such as caspase 8 [34].

4.2 Induction of Cyp26a1 and generation of 4-oxo-FEN

Results from ovarian carcinoma cells also demonstrated that endogenous *Crbp1* and *Cyp26a1*gene expression was elevated >20-fold when continuously treated with FEN compared to non-treated cells [35]. Similarly to RA, the oxidation of FEN via the induction of *Cyp26a1* can result in the generation of polar metabolites. One metabolite, N-(4-hydroxyphenyl)-4-oxoretinamide (4-oxo-FEN), has been identified in both FEN treated ovarian carcinoma cells and plasma from patients participating in a FEN clinical trial [35]. 4-oxo-FEN levels were also detected when RARβ and RARγ were overexpressed, indicating the

involvement of the canonical retinoid signalling in the generation of 4-oxo-FEN [35]. As observed with the parent compound FEN, 4-oxo-FEN was found to be more effective at inhibiting proliferation of numerous tumour cell lines [36]. Although 4-oxo-FEN is generated through activation of the retinoid metabolism pathway, the anti- proliferative action of 4-oxo-FEN was proposed to be through RAR-independent mechanisms since, 4-oxo-FEN was able to inhibit cancer cell proliferation in both FEN-sensitive and FEN-resistant cell lines. Additionally, 4-oxo-FEN was observed to bind poorly to RARs and RAR antagonist treatment failed to prevent 4-oxo-FEN mediated cell growth inhibition [35]. Moreover, unlike FEN and independent of ROS generation, 4-oxo-FEN also appears to cause G2-M mitotic arrest through anti-microtubule activities [36]. However, there are a limited number of studies with FEN metabolites and thus it is not clear whether they share common or very different mechanisms of action with FEN.

5. RAR-independent mechanisms of FEN-induced apoptosis

In most cell systems, the apoptotic effect of FEN appears to be independent of RAR activation and involves generation of ROS and lipid second messengers [21]. Most consistently, antioxidants (e.g. vitamin C, N-acetylcysteine and butylated hydroxyanisole) have been shown to inhibit FEN-induced apoptosis. Early studies in various cancer cells indentified that FEN-induced apoptosis was associated with sustained-activation of mitogen activated protein kinase (MAPK)s JNK, p38 and ERK1/2, induction of proapoptotic transcription factor GADD153/CHOP and BCL-2 family member BAK and downstream activation of caspase-9 and caspase-3 [21]. The induction of sphingolipid second messenger ceramide and ganglioside GD3 by means of *de novo* synthesis via ceramide and GD3 synthases and/or hydrolysis of sphingomylin and downstream activation of 12-lipoxygenase has also been postulated to be a mechanism of FEN-mediated induction of BAK and

GADD153/CHOP leading to apoptosis by Lovat, Redfern and co-workers [21]. Due to the constraints (maximum word and reference limits) at use in this journal for its short reviews, we have chosen to focus on the more recent developments in the signalling pathways proposed for FEN-induced apoptosis or cell survival. Earlier studies from the 1990s to mid-2000s (as briefly mentioned in this paragraph) are well documented in the 2006 review by N. Hail Jnr *et al*. [21]. The high level of cross-talk between the multiple signalling pathways postulated to play a role in FEN-mediated biological effects are summarised in Fig. 2.

5.1 Induction of pro-apoptotic BAK versus anti-apoptotic Bcl-2

All of the pro-apoptotic effects of FEN, including ROS generation, have been shown to require induction of pro-apoptotic Bak in neuroblastoma cells and to be suppressed in cervical cancer cells with the overexpression of anti-apoptotic Bcl-2. Thus, in a strategy to inhibit Bcl-2 family members in combination with FEN, Reynolds and co-workers found that ABT-737, a small-molecule BH3-mimetic that inhibits most proteins of the Bcl-2 family, could enhance FEN activity in neuroblastoma [37]. FEN in combination with ABT-737 induced greater mitochondrial membrane depolarization and mitochondrial cytochrome c release, greater activation of caspases of both the intrinsic and extrinsic pathways, greater activation of Bax-α, t-Bid, and Bak, and a higher level of apoptosis than either drug alone. *In vivo*, FEN with ABT-737 showed a similar anti-neuroblastoma activity in a mouse xenograft model of neuroblastoma. Thus, the synergistic cytotoxic effects of drug combination of FEN with an inhibitor of Bcl-2 family members hold great prospects and warrants future clinical trials.

5.2 ROS production via mitochondrial electron transport chain

Since FEN-induced ROS production could be decreased in intact cells co-treated with rotenone or certain co-enzyme Q analogues, this implied that the turnover of complex I may contribute to the pro-oxidant activity of FEN [21]. State-of-the-art experimental methodologies utilising isolated mitochondrial preparations with respect to establishing the direct mitochondrial toxicity of agents like FEN, still have their limitations and may require additional validation in a cellular context. Consequently, the direct and/or indirect mitochondrial effects of FEN may be challenging to elucidate fully. However, it is certainly possible that FEN could promote ROS at a site associated with oxidative phosphorylation that is specifically required in rapidly dividing cells such as transformed cells, and not by disrupting oxidative phosphorylation in general which would produce far more adverse side effects than those commonly observed.

Hail and co-workers recently hypothesised that dihydroorotate dehydrogenase (DHODH), an enzyme associated with mitochondrial electron transport and required for *de novo* pyrimidine synthesis, could be an important link between mitochondrial bioenergetics, cell proliferation, and sensitivity to FEN-induced ROS and apoptosis in certain transformed cell types [38]. In prostate and skin cancer cells the suppression of DHODH activity by chemical inhibition or the reduction in DHODH protein expression by RNA interference markedly decreased FEN-induced ROS generation and apoptosis. Conversely, colon carcinoma cells that lacked DHODH expression were markedly resistant to the pro-oxidant and cytotoxic effects of FEN. This study strongly implicates DHODH in FEN-induced ROS production and apoptosis.

5.3 Dihydroceramide generation & autophagy induction

Early studies had shown FEN-induced increases in ceramide, however analysis by liquid chromatography-tandem mass spectrometry (LC-MS) has determined with more

specificity that FEN is responsible for increased levels of dihydroceramide [39], the immediate precursor of ceramide. FEN was shown to inhibit dihydroceramide desaturase activity in cell-based and *in vitro* assays [40]. It was also shown in this study that RA failed to inhibit dihydroceramide activity, indicating that FEN acted in a RAR-independent manner to increase dihydroceramide levels. Kraveka and co-workers have gone on to show more recently that FEN (and 4-oxo-FEN) can act as a direct inhibitor of the enzyme dihydroceramide desaturase 1 (DES1) *in vitro* [41]. This enzyme is responsible for the desaturation of dihydroceramide, final step of *de novo* synthesis of ceramide lipid species from dihydroceramide precursors. Thus inhibition of DES1 would prevent the final step in the production of ceramide and lead to an accumulation of dihydroceramide.

Further LC-MS analysis of sphingolipids in several cancer cell lines has identified that treatment with either FEN or 4-oxo-FEN leads to a marked increase in dihydroceramide and complex dihydrosphingolipids, while only 4-oxo-FEN led to a minor increase of ceramide species [42,43]. These findings are of considerable interest since dihydroceramides are thought to be biologically inert thereby they are inactive on the pathways modulated by ceramides, but it has recently been reported that dihydroceramides can induce autophagy in prostate cancer cells and cell growth inhibition with cell cycle arrest in neuroblastoma cells [39,40].

5.4 mTOR and autophagy, a cell survival mechanism.

The mechanism(s) by which FEN can lead to the induction of autophagy and/or apoptotic cell death is currently unclear. Both FEN exposure and dihydroceramides accumulation can initiate cellular survival pathways such as the ER stress response and autophagy induction [39,44-46]. FEN has also been reported to inhibit the kinase activity of mammalian target of rapamycin (mTOR) both *in vitro* and *in vivo* [47]. This may possibly

occur through the direct binding of FEN to the ATP pocket of mTOR based on computer modelling of the crystal structure of PI3K-delta [47]. Since mTOR is a key inhibitor of autophagy, inhibition of mTOR by FEN may result in an increase in autophagy induction.

Autophagy plays an important role in cell survival as its inhibition in mammalian cells during nutrient depletion causes apoptosis. Interestingly, the presence of 10% serum in cell culture media strongly abrogated FEN-mediated apoptosis [48]. Moreover, FEN treatment at suboptimal doses for apoptotic induction was shown to induce autophagy and proposed to act as survival advantage to malignant glioma cells [49].

5.5 ROS induced cytoxicity independent of ceramide and autophagy

In human pancreatic cancer cells, FEN-induced cytotoxicity appears to be mediated by ROS, but not by ceramide, since antioxidants and autophagy inhibitors (but not *de novo* ceramide inhibitor myriocin) blocked FEN-induced LC3 II expression and partially inhibited cell death [50]. Asumendi and co-workers found similar results in leukemia cells, suggesting that the two hallmarks of FEN-mediated cell death are independent mechanistic events [51].

5.6 ROS, DJ-1, ASK1, p38 apoptosis pathway

Interestingly, FEN-induced activation of the c-Jun N-terminal kinase (JNK) and p38 MAPK in several cancer cell lines has been shown to be suppressed by antioxidants. Moreover, FEN-induced apoptosis is decreased by down regulating JNK or p38 MAPK activity using chemical inhibitors or small interfering RNAs [21]. JNK and p38 MAPK are activated by a wide range of cellular stresses including ROS. Recent findings in Hela cells have now implicated DJ-1, a multifunctional oxidative stress response protein and the ASK-1-p38 MAPK pathway to regulate the balance between autophagy and apoptosis depending on the relative concentration of FEN and subsequent level of ROS generation [22]. ASK1-mediated activation of JNK and p38 were found to be responsible for the FEN-induced

autophagy or apoptosis, respectively. However, the mildly oxidised form of DJ-1 (in the presence of a low FEN concentration) was found to bind to and inhibit ASK1 activation of p38 and thus inhibit FEN-induced apoptosis (via ROS generation). Moreover, this promoted FEN-induced autophagy and cell survival. Increasing the FEN concentration to induce high levels of ROS caused excessive DJ-1 oxidation and dissociation from ASK1, leading to p38 activation and apoptosis. Promisingly, DJ-1 depletion *in vivo* with shRNA enhanced the sensitivity of tumor cells to FEN [22].

5.7 Hypoxia and HIF-1alpha

Hypoxia induces resistance to many forms of anti-cancer therapy including FEN [52,53]. Moreover, under hypoxic conditions, FEN-induced autophagy appears to be hypoxia-inducible factor (HIF)-1-α dependant and not inhibited by antioxidants [54]. Knockdown of HIF-1α inhibited autophagy but promoted 4-HPR-induced apoptosis suggesting an alternative strategy to overcome resistance to FEN-induced anti-cancer activity. There is a considerable body of evidence now, independent of studies with FEN, that implicates autophagy as a mostly cytoprotective mechanism and that it rarely, if ever, constitutes a lethal effector mechanism that is responsible for cell death [55].

5.8 MIC-1/PLAB/ NAG-1/ GDF-15

To identify novel genes contributing to its apoptotic activity in ovarian cancer cells, transcriptome profiling was performed in human ovarian carcinoma cells and human umbilical vein endothelial cells. Macrophage inhibitory cytokine-1 (MIC-1), a proapoptotic and antiangiogenic gene, was the most highly induced [56,57]. MIC-1 levels were highly associated with FEN-induced apoptosis in several cell lines and were also induced in ascitic cells collected from patients with ovarian cancer before and after FEN treatment., The ER stress inhibitor salubrinal and the antioxidant vitamin C, abrogated 4HPR-induced activation

of JNK, MIC-1 up-regulation and protected the cells from apoptosis [58]. These results indicate a role for MIC-1 as a mediator of FEN-induced apoptosis at least in certain ovarian cancer cell lines. MIC-1 encodes a protein sharing homologies with members of the transforming growth factor (TGF)-β superfamily and is also known as non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1), PLAcental Bone morphogenetic protein (PLAB), placental-TGFβ, prostate-derived factor (PDF) and growth differentiation factor-15 (GDF-15). Studies with transgenic mice expressing human MIC-1 demonstrated that increased MIC-1 levels can inhibit the development of some tumors in animal models. However, contrasting laboratory and clinical evidence suggests that MIC-1 probably has diverse functions in carcinogenesis [59]. Interestingly, tumor-induced anorexia and weight loss may be partly mediated by overproduction of MIC-1 by tumors [60] (see following sections on the regulation of glucose and lipid homeostasis by FEN).

The high level of cross-talk between these signalling intermediates has to date made it extremely difficult to elucidate exactly which genes and pathways are required for FEN's biological activities. ROS-mediated stress kinase activation appears to be central to FEN-induced apoptosis where the novel discoveries regarding DHODH and DJ-1 may be critical missing links. In contrast, inhibition of DES1 leading to elevations in dihydroceramide levels is probably cytoprotective via promotion of autophagy pathways. The multiple signalling pathways involved in FEN-mediated anti-cancer activity in particular RAR-independent mechanisms of FEN-induced apoptosis are summarised in Fig. 2.

6. Regulation of glucose and lipid homeostasis by FEN.

6.1 Lowering of circulating levels of RBP4 and retinol levels.

In the first FEN human anti-cancer trials, FEN treatment was found to induce a decrease of plasma retinol, which was associated with an impaired adaptation to dark [17]. This side-effect could be minimized with a 3-day treatment interruption per month to increase plasma retinol concentrations and partial recovery of retinoid storage. This initial finding has contributed to one of the characteristic effects of FEN treatment, to lower the circulating levels of the specific retinol transport protein RBP4 [61]. RBP4, is primarily synthesised in the liver but also adipose tissue. Its primary function is to transport retinol (hydrolysed from stored retinyl esters) to supply peripheral tissues via tight binding to this specific serum transport protein [7,62]. Due to its small size (21 kDa), the retinol-RBP4 complex is prone to glomerular filtration but binding with another serum protein transthyretin prevents its loss from the circulation. FEN has a high binding affinity for RBP4 and thus can disrupt the complex [61]. FEN has been shown to form a tight association with RBP4 and the FEN-RBP4 complex has been detected by immunoprecipitation [29,61]. FEN therefore maintains the ability to bind RBP4, but due to the presence of the bulky 4-hydroxyphenyl group, the protein-protein interaction between RBP4 and TTR is prevented from forming. Confirmation that this occurs was provided when the FEN-RBP4 complex obtained from treating the human hepatoma cell line (HepG2) with FEN, displayed a decrease in binding to a TTR affinity column [63]. Thus, by preventing the formation of the RBP4-TTR complex and increasing glomerular filtration after treatment with FEN, this leads to elevated levels of RBP4 in the kidney and urine and subsequently lowering of circulating levels of RBP4 and retinol [64]. It is this characteristic mechanism which first resulted in the application of FEN treatment to prevent insulin resistance associated with high-fat diet (HFD) feeding in mice.

In 2005, important findings originating from the laboratory of B.B. Kahn described the altered gene expression of RBP4 specifically from adipocytes and the negative contribution of elevated serum levels in the regulation of insulin sensitivity [65]. Elevated levels of this 'adipokine' were found to associate with insulin resistance in multiple models of obesity and type II diabetes. FEN was identified as a potential pharmacological means of intervention and thus, by the mechanisms described above, FEN treatment provided an opportunity to decrease the elevated serum levels of RBP4 observed in HFD-induced states of obesity and insulin resistance. FEN decreased the elevated serum levels of RBP4 found in obese mice that had been fed a HFD, which subsequently led to improvements in insulin sensitivity [65]. These findings along with additional elegant experiments to genetically or pharmacologically increase circulating RBP4 levels provided evidence for a role for elevated RBP4 levels in impaired glucose homeostasis, which FEN was able to attenuate. It was reported in these initial investigations (which lasted for up to sixteen weeks in the FVB strain of mice), that FEN treatment did not affect food intake or bodyweight levels with HFD feeding. Importantly, since the discovery of FEN's additional beneficial effects in preventing insulin resistance in mice, it is also currently in a Phase-II clinical trials at the University of San Diego (California, USA) for the treatment of insulin resistance and liver inflammation related to non-alcoholic fatty liver in obese humans with results to be posted in early 2015.

6.2 Mechanisms independent of RBP4 lowering.

A follow up examination provided detailed physiological evidence that the chronic treatment of mice with FEN was also able to partially prevent the onset of HFD-induced adiposity and obesity. These findings in FVB mice were apparent with the use of both a preventative and interventional approach [13]. Intriguingly, the beneficial anti-obesity effects observed with FEN treatment were entirely reproducible in mice lacking RBP4, i.e. genetically null animals (*Rbp4*$^{(-/-)}$ mice) on the C57/129Sv mixed background, first described

by L. Quadro and colleagues [62]. This implied that the mechanism by which FEN functions to reduce body weight and adiposity was likely to be independent of the ability of FEN to reduce circulating levels of RBP4. Furthermore, not all models of obesity, insulin resistance or type II diabetes have reported elevations in circulating RBP4 levels however, technical problems using enzyme-linked immunoassays may undervalue elevated serum RBP4 concentrations [66].

6.3 Fen-induced RA-like effects on energy balance and glucose homeostasis

Although it was documented that FEN could prevent the onset of HFD-induced gain in total body mass and more specifically fat mass, extensive examination revealed that FEN did not lead to measurable changes in food intake, energy expenditure, physical activity or stool lipid content [13,14]. Moreover, although RA also induces mitochondrial uncoupling protein (UCP)-1 in BAT to increase energy expenditure [67], FEN did not increase UCP1 levels in BAT or WAT [14].

However, FEN did inhibit HFD-induced elevation in leptin serum levels and directly inhibited leptin mRNA in fully differentiated adipocytes [14]. Moreover, in a breast cancer clinical trial in premenopausal women, FEN improved insulin sensitivity and decreased serum leptin levels specifically in overweight women [68]. Leptin is released from adipocytes in postprandial states and acts as a satiety hormone via hypothalamic pathways to reduce food intake and increase energy expenditure. Leptin secretion is positively correlated with adiposity and therefore during states of obesity, circulating leptin levels are increased [69]. In obesity, this elevation is associated with loss of leptin-mediated action termed leptin resistance which ironically perpetuates obesity further. Interestingly, RA has been shown to decrease body weight and adiposity and to target leptin via decreases in WAT mRNA expression along with its secretion [67].

FEN-mediated alterations in adipose gene expression were not limited to leptin. FEN treatment prevented HFD-induced downregulation of peroxisome proliferator-activated receptor (*Ppar*)-*γ*, glucose transporter (Glut)-4 and *adiponectin* and lowered serum resistin and RBP4 levels [14]. Furthermore, both long term (20 weeks) and short term (7 days) FEN treatment lead to a marked induction in classic retinoid-responsive genes *Crbp1*, *Rarβ* and *Cyp26a1* suggesting RAR-signalling was responsible for FEN's effects.

Hepatic level of rate-limiting gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) is hormonal regulated during fasting and feeding. PEPCK is also induced with RA or RBP4 treatment [65,70] or decreased with substantially impaired RA synthesis in retinaldehyde dehydrogenase (RALDH)-1 knockout mice [71]. These studies strongly support retinoid nuclear receptor-mediated effects on PEPCK as a key determinant of hepatic gluconeogenesis and glucose intolerance associated with obesity and insulin resistance. However in contrast, euglycemic-hyperglycemic clamp studies in HFD-obese mice, FEN treatment completely normalised suppression of hepatic glucose production by insulin in association with improved whole body and skeletal muscle glucose uptake [13]. These findings imply that FEN-induced RAR-signalling in liver does not lead to induction of PEPCK and increased levels hepatic gluconeogenesis with HFD-induced obesity *in vivo*.

Normalisation of HFD-induced hyperglycemia with FEN treatment maybe partly through central/hypothalamic effects of improved leptin sensitivity on the regulation of hepatic glucose production via the autonomic nervous system [13,14,72]. Interestingly, central administration of orexigenic neuropeptide Y (NPY) has been shown induce hepatic insulin resistance and RA can downregulate NPY in neuroblastoma cells [73,74]. Thus, a second possible central mechanism for the improved glucose homeostasis could be via direct suppression of NPY expression in the hypothalamus of FEN-treated mice [14].

As discussed earlier (section 5.8), MIC-1 was identified by transcriptome profiling of ovarian carcinoma cells as highly induced by FEN and associated with FEN-induced apoptosis in several cell lines [57]. Thus, FEN-induced MIC-1 up-regulation may mediate some of the anti-obesity effects of FEN treatment since overproduction of MIC-1 by tumors has been reported to contribute to tumor-induced anorexia and weight-loss in mice [60]. These studies identified MIC-1-signalling via hypothalamic TGFβ receptor II, ERK1/2 and signal transducer and activator of transcription (STAT)-3 led to up-regulation of POMC anorexigenic and downregulation of NPY orexigenic pathways, similar to the pattern observed with weight-loss in leptin-treated animals. However, it is not currently known if MIC-1 is induced by FEN in obesity models.

While there are studies supporting an anti-adiposity action and overall beneficial effect of RA on metabolic profile, including changes in hepatic lipid metabolism leading to repartitioning of fatty acids away from triacylglycerol storage and towards oxidation, retinoid-induced hypertriglyceridemia is a relatively frequent side effect of retinoid therapy (e.g. for dermatological disorders) [67]. Retinoid-induced hypertriglyceridemia in humans has also been modelled in a number of rodent studies and has been reported to occur in response to high doses of vitamin A (as retinol or retinyl palmitate), RA isomers (including RA, 9-cis RA and 13-cis RA), and synthetic RXR-specific agonists (rexinoids) [67]. Importantly, FEN-treatment decreased severe hepatic steatosis by 50% in HFD-obese mice and did not increase circulating triglycerides, free-fatty acids or glycerol [13,14].

6.4 RA-signalling inhibits adipocyte differentiation.

Adipogenesis is a complex and temporally regulated signalling cascade, which generates the machinery required in order for adipocytes to take up substrates for the synthesis and safe storage of lipids as triacylglycerols [75]. Confluent pre-adipocyte

(fibroblast-like) cell cultures can be synchronously induced to differentiate with an adipogenic "cocktail" stimulating glucocorticoid, cyclic-AMP and insulin-signalling. Numerous transcription factors are then induced and participate during the programme that is instrumental for terminal differentiation to occur. The most well-characterised of these are members of the CAAT/enhancer-binding protein (C/EBP) family of transcription factors, *C/ebp beta* and *C/ebp delta* which are induced early and transiently during adipocyte differentiation. Followed by induction of two intermediate and crucial regulators of adipogenesis, C/EBPα and PPARγ, of which PPARγ is the key master regulator of adipogenesis.

Consistent with the findings that alterations to the retinol metabolism pathway play an important role in the regulation of adiposity, it has been well established that RA-RAR signalling is able to inhibit pre-adipocyte models of adipogenesis [76,77]. Detailed experimental evidence has revealed that the ability of RA to inhibit adipocyte differentiation is temporal, with RA inhibition only being achieved when supplemented within twenty-four hours of initiation of adipocyte differentiation [76,78]. This loss of RA inhibition occurs due to the downregulation of RARs, which is observed during adipocyte differentiation [78]. Consistent with this view, the RA window of inhibition can be extended up to forty-eight hours with the overexpression of RAR subtypes. However, after this time it appears adipocyte conversion reaches an irreversible check point where RA is no longer able to have an effect [78]. Additionally, the inhibition of adipocyte differentiation by RA was shown to be caused by the prevention of C/EBPβ mediated transcriptional activation [79]. In these studies, RA did not block the transcriptional induction of *C/ebp beta*, but inhibited its downstream induction of PPARγ and C/EBPα, which subsequently prevented expression of terminal adipocyte markers and the conversion of pre-adipocytes into mature lipid laden cultures. Results obtained in our lab, suggest that FEN acts similarly to RA in 3T3-L1 cells by blocking

adipogenesis via inhibition of C/EBPβ-mediated transcription of PPARγ, C/EBPα and subsequently expression of terminal adipocyte markers [14].

Members of the AP1 family of transcription factors are induced immediately after the induction of adipocyte differentiation. RA can also downregulate the transcriptional activation of AP1 [80] and therefore may prevent early cell cycle events during the induction of the adipogenic transcription cascade. In transrepression assays in Hela cells co-transfected with RARs, a relatively high concentration of FEN (20 μM) was also found to be a potent inhibitor of AP1, suggesting another target of FEN-mediated inhibition of adipogenesis [31].

Further investigations have indicated that RA does not directly prevent C/EBPβ-mediated transcriptional itself, but does so through increasing levels of a transcription factor from the mothers against decapentaplegic homolog (SMAD) family. Increased levels of SMAD3 were shown to interact with C/EBPβ and interfere with its ability to occupy the C/EBPα promoter. Moreover, in the absence of SMAD3, RA is no longer able to inhibit adipocyte differentiation [81]. It is currently unknown if FEN can also alter SMAD3 levels.

RA or FEN-treatment leads to marked up-regulation of *Crbp1* in adipocytes and in carcinoma cells [14,35]. Interestingly, in *Crbp1*$^{(-/-)}$ mouse embryonic fibroblasts differentiated into adipocytes, or 3T3-L1 adipocytes where CRBP1 had been knocked down revealed increased triacylglyceride accumulation due to increased expression and activity of PPARγ. The overexpression of CRBP1 in 3T3-L1 cultures resulted in significantly reduced levels of triacylglyceride compared to controls [82]. These results suggest that CRBP1 can either directly influence PPARγ activity or do so indirectly by regulating retinoid homeostasis and RAR-signalling.

6.5 Involvement of the non-canonical retinoid signalling pathway.

Noy and co-workers have shown that adipocyte differentiation is accompanied by downregulation of RAR and CRABP-II and upregulation of PPARβ/δ and FABP5. Consequently, whereas in preadipocytes RA functions predominantly through CRABP-II and RAR, the hormone signals through both pathways in the mature adipocytes [83]. Multiple studies established that RA treatment results in weight loss and enhances insulin sensitivity in various mouse models of obesity [83,84]. These effects can be traced, at least in part, to enhanced fatty acid oxidation and energy dissipation brought about by RA-induced activation of PPARβ/δ and RAR in mature adipocytes, liver, and skeletal muscle [83,85]. It is not currently known whether FEN can signal via PPARβ/δ.

6. 6 Induction of apoptosis as a potential mechanism.

The extensive investigation of FEN has largely been due to the early discovery that it displayed favourable properties as a chemopreventive agent for breast cancer [11,17]. Subsequent studies revealed that FEN was able to attenuate uncontrolled cell proliferation in multiple cancer cell lines through the induction of apoptosis [21]. Seeing as FEN accumulates in fatty tissue and prolonged treatment prevents HFD-induced adiposity, it could be hypothesised that FEN may lead to the induction of adipocyte apoptosis and thereby lead to decreased adiposity. Through mechanisms that are not well established, FEN is able to cause apoptosis in cancerous transformed cells but not in normal cells which are unaffected by similar concentrations of FEN treatment [21]. Consistent with the view that FEN does not induce apoptosis in non-transformed cells, no alteration in the number of subcutaneous-WAT adipocytes was found in both the preventive and interventional studies, where FEN completely prevented subcutaneous-WAT mass expansion [13]. Although not conclusive evidence, these findings indicate that FEN is unlikely to cause apoptosis in developed adipose tissue and alternative mechanisms are therefore expected to be involved. Reports of hypoxia and HIF1α up-regulation in obesity [86] may actually protect adipocytes from FEN-induced

apoptosis, similar to the mechanism of hypoxia-induced resistance to anti-cancer therapy (section 5.7).

6.7 Potential RAR-independent mechanisms of Fenretinide.

Interestingly, preventing ceramide lipid species accumulation may provide an alternative RAR-independent mechanism by which FEN operates to prevent the negative effects of HFD feeding on glucose and lipid regulation. Increased ceramide synthesis in response to excessive glucocorticoids, saturated free fatty acids or tumour necrosis factor (TNF)-α is associated with an inhibition of insulin signal transduction by promoting the dephosphorylation of Akt/PKB by protein phosphatase 2A (PP2A) and by blocking the activation and translocation of Akt/PKB from the cytoplasm to the plasma membrane [87,88]. Moreover, inhibition of ceramide synthesis improves glucose homeostasis in rodent models of obesity and insulin resistance. Specifically, genetic knockout of DES1, or treatment with *de novo* ceramide inhibitor myriocin improves glucose tolerance in rats [89]. In association with improved skeletal muscle and hepatic insulin sensitivity *in vivo*, myriocin pretreatment lowered ceramide levels and improved insulin action at the level of Akt/PKB. Thus, from these studies it could be concluded that ceramide-induced inactivation of Akt/PKB is a contributing mechanism by which the sphingolipid impairs insulin action [89].

Thus, by altering rates of cellular ceramide production at the level of DES1, FEN has been shown to prevent lipid induced insulin resistance in both cultured myotubes and isolated muscle strips [90]. Additionally, it was observed *in vivo* that increases in dihydroceramide levels were present in HFD fed mice treated with FEN incorporated into the drinking water. These alterations were associated with improvements in glucose homeostasis [90]. In these studies, FEN was found not to have an effect on adiposity. It is currently unknown if FEN also alters dihydroceramide levels in adipose tissue, which could be mechanistically

responsible for the beneficial outcomes when mice are supplemented with FEN in background of obesity. Since FEN exposure and dihydroceramide accumulation can initiate autophagy induction, FEN-mediated increases in dihydroceramide levels may activate a potential RAR-independent mechanism of FEN action in vivo.

Defective autophagy has been shown to play a role in hepatic insulin resistance during states of obesity [91]. Furthermore, autophagy appears to be involved in pancreatic beta-cell compensation during periods of HFD feeding [92]. The role of defective autophagy in adipose tissue has not been an area extensively investigated and so its function is currently unclear. Obese individuals for example have increased markers of autophagy in adipose tissue [93], which has led to the speculation that increased levels of adipose tissue autophagy may facilitate adipocyte enlargement [94]. Other studies however suggest that hypertrophic adipocytes display increased levels of autophagy due to the accumulation of autophagosomes that have not been appropriately processed due to reduced autophagic flux [95]. Whether FEN is able to alter levels of autophagy in adipose tissue has not been investigated to date. This however could provide an additional mechanism by which FEN modulates in order to inhibit adipose expansion and the development of insulin resistance in mice. The pathways proposed to play a role in FEN-mediated improvements in whole body metabolic homeostasis are summarised in Fig. 3.

7. Conclusions and future directions.

The mechanism of FEN action to induce apoptosis in cancer models and to prevent diet-induced obesity and insulin resistance in mice has been under investigation for the last 20 years or so. The pathways involved include ROS and dihydroceramide generation and the activation of stress kinases and autophagy (summarised in Fig. 2). Although the RAR-dependant effects of FEN have been largely ignored by the cancer field for the last 10 years,

the growing interest in vitamin A as a modulator of body fat mass and glucose homeostasis has highlighted nuclear hormone receptor signalling as important once again in mediating at least some of the beneficial actions of FEN (summarised in Fig. 3). How these pathways may interact in different tissues, in different disease models and under various experimental conditions remains to be elucidated. The exact mechanism of altered nuclear hormone signalling (particularly in adipose tissue) to induce these beneficial actions also remains an unanswered question. However, given the safe toxicological profile of this synthetic retinoid, it would appear to be of relatively high clinical importance to continue to investigate the mechanism(s) of FEN action in specific cells, tissues and at the whole organism level. Delineating these should provide further rationale for improving the efficacy of FEN action to (1) induce apoptosis in cancerous tissues, (2) prevent obesity or (3) improve glucose homeostasis in obesity and type-2 diabetes. This may be in synergy with other chemotherapeutics or anti-obesity/diabetic regimens or through the development of improved analogues of FEN.

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Figure legends

Fig. 1. The chemical structure of all-*trans*-retinoic acid and N-(4-hydroxyphenyl)retinamide. FEN is identical to RA except for the modification to the carboxyl functional group, which is replaced with an amide linked 4-hydroxyphenyl group.

Fig. 2. Signalling pathways proposed for FEN-induced apoptosis or cell survival. Refer to main text in sections 4 and 5 and [21] for details. Abbreviations: RAR, retinoic acid receptor; RARE, retinoic acid response element; Nurr77, a.k.a. nuclear receptor subfamily 4, group A, member 1 (NR4A1) or orphan nuclear receptor T3 (TR3); Bcl-2, B-cell CLL/lymphoma 2, founding member of the apoptosis regulator proteins; OXPHOS, oxidative phosphorylation; DHODH, dihydroorotate dehydrogenase; ROS, reactive oxygen species; PERK, protein kinase activated by double-stranded RNA (PKR)-like endoplasmic reticulum kinase; eIF2α, eukaryotic initiation factor 2α; CHOP, CCAAT-enhancer-binding protein (C/EBP) homologous protein also known as growth arrest and DNA damage-inducible gene 153 (GADD153); BAK, BCL2-antagonist/killer; DJ-1, multifunctional oxidative stress response protein a.k.a. Parkinson disease protein 7 (PARK7); IRE1, inositol-requiring enzyme 1; ASK1, apoptosis signal-regulating kinase 1; p38, mitogen-activated protein kinase (MAPK) family member; JNK c-Jun N-terminal kinase; MIC-1, macrophage inhibitory cytokine-1 a.k.a. non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1), PLAcental Bone morphogenetic protein (PLAB), placental-TGFβ, prostate-derived factor (PDF) and growth differentiation factor-15 (GDF-15); DES-1 dihydroceramide desaturase 1 is the final step of *de novo* synthesis of ceramide lipid species; Akt, a.k.a. protein kinase B (PKB); HIF-1α, hypoxia-inducible factor 1α; mTOR, mammalian target of rapamycin.

Fig. 3. Molecular pathways proposed for FEN-mediated improvements in whole body metabolic homeostasis. Refer to main text in section 6 for details. The exact mechanism of altered nuclear hormone signalling (particularly in adipose tissue) to induce these beneficial actions remains a major unanswered question (marked by ? in the figure). Abbreviations: RAR, retinoic acid receptor; RARE, retinoic acid response element; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT-enhancer-binding protein α; RBP4, serum retinol binding protein; GLUT4, glucose transporter 4; NPY, neuropeptide Y; DES-1 dihydroceramide desaturase 1 is the final step of *de novo* synthesis of ceramide lipid species; Akt, a.k.a. protein kinase B (PKB).

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Figure

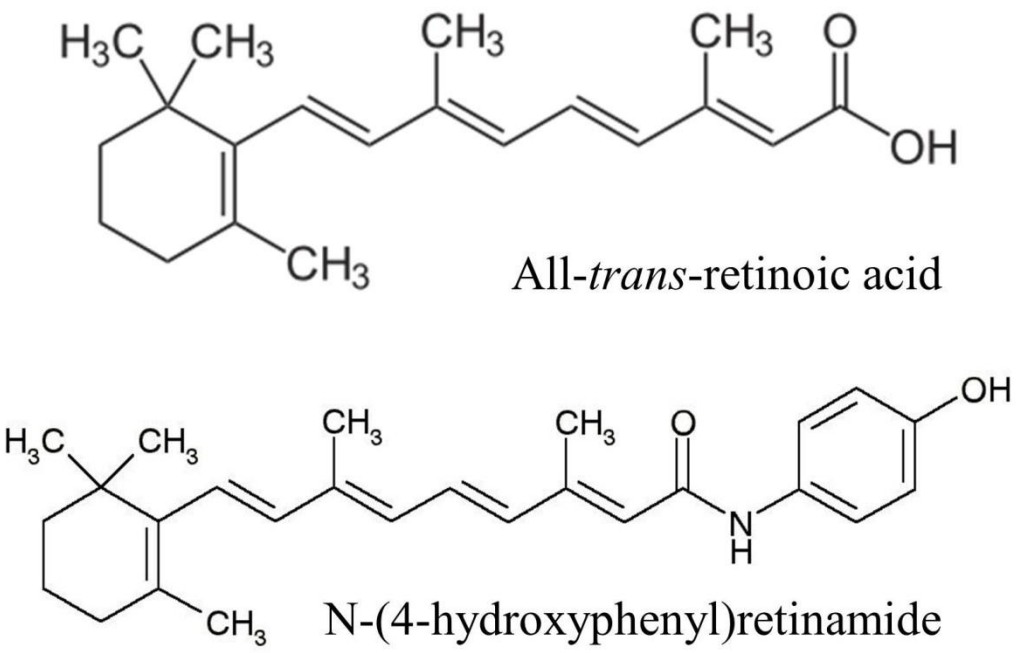

Fig. 1. The chemical structure of all-*trans*-retinoic acid and N-(4-hydroxyphenyl)retinamide. FEN is identical to RA except for the modification to the carboxyl functional group, which is replaced with an amide linked 4-hydroxyphenyl group.

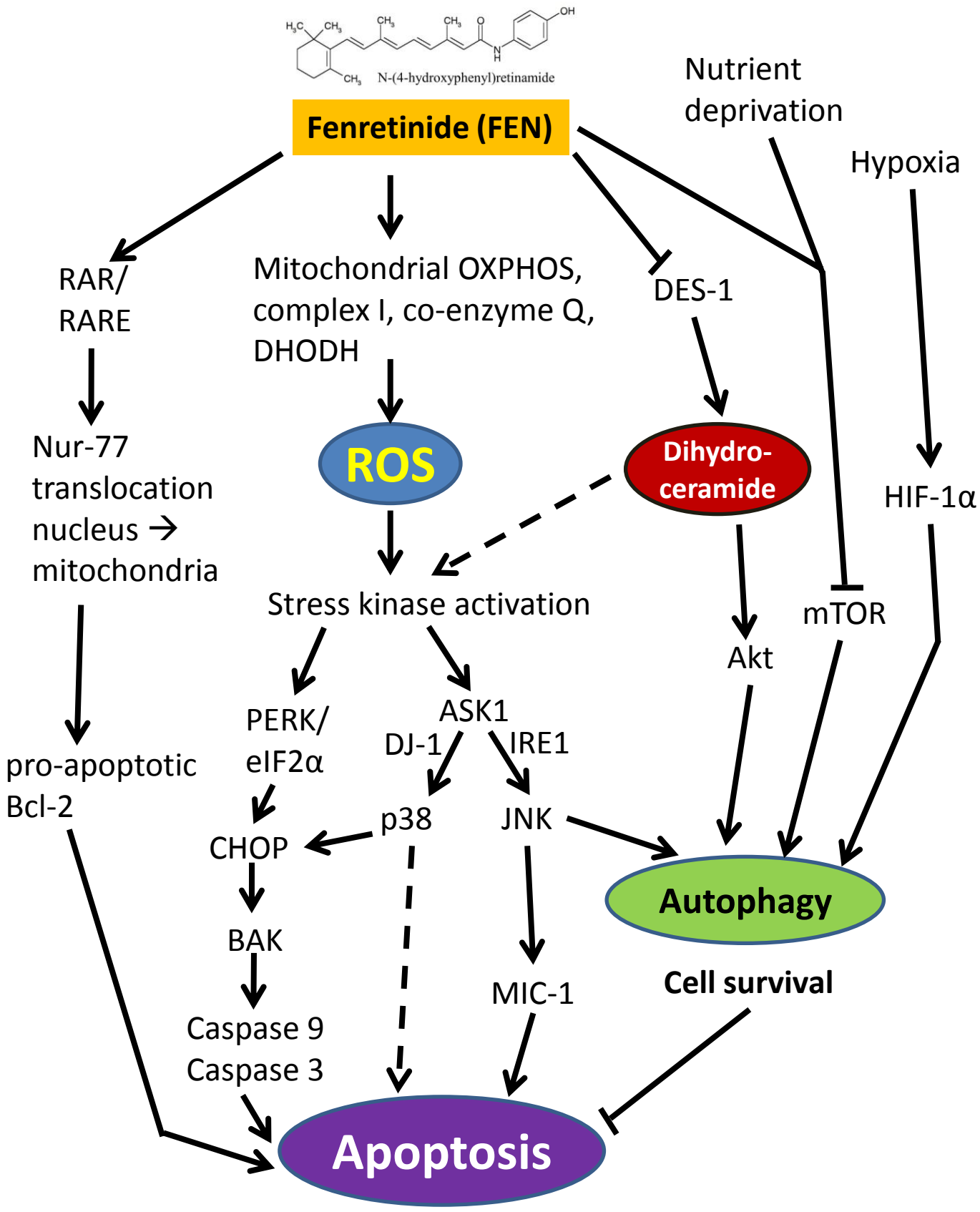

Fig. 2. Signalling pathways proposed for FEN-induced apoptosis or cell survival. Refer to main text for details.

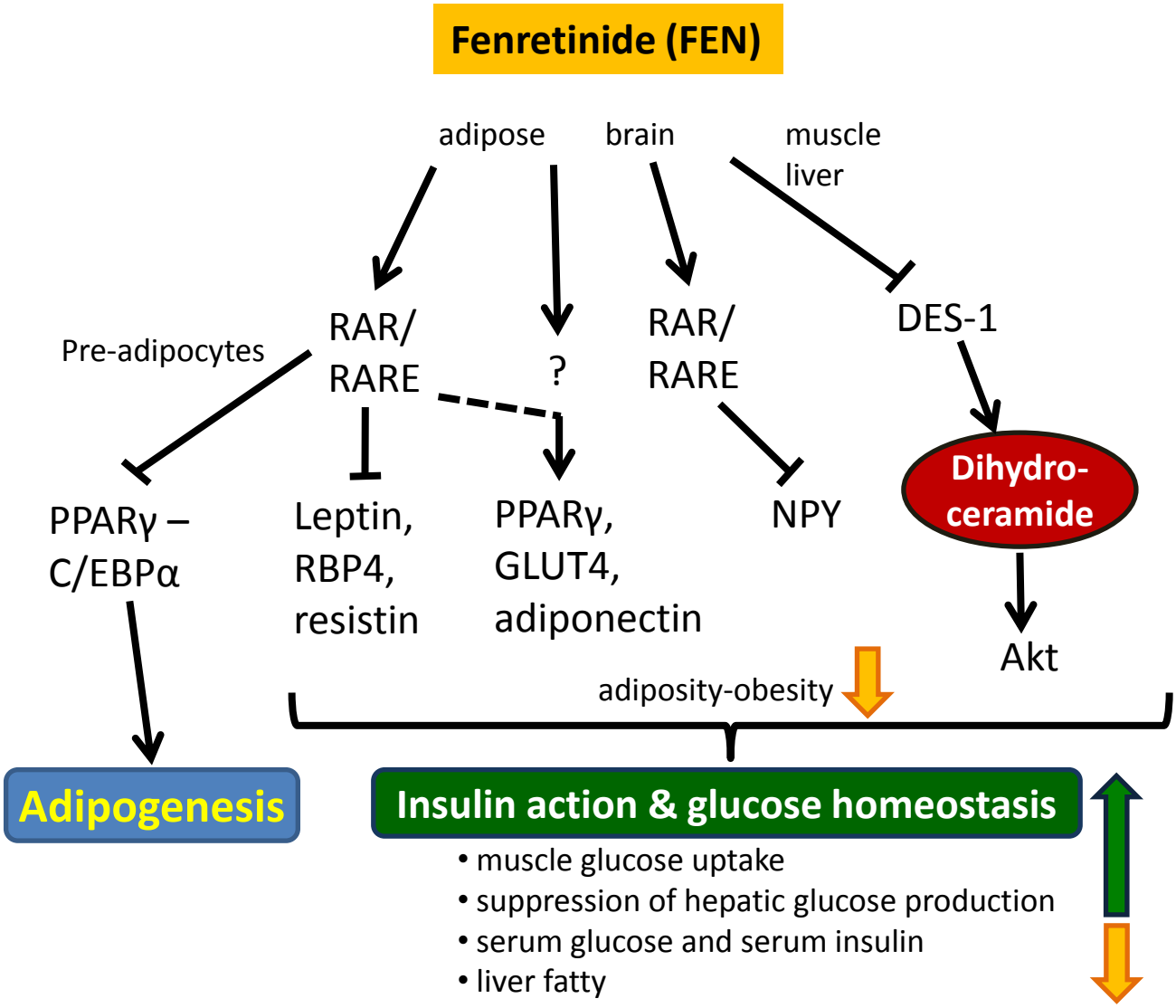

Fig. 3. Molecular pathways proposed for FEN-mediated improvements in whole body metabolic homeostasis. Refer to main text for details.