The role of the 14-3-3 protein family in health, disease, and drug development

Yasaman Aghazadeh[1,2] and Vassilios Papadopoulos[1,2,3,4]

[1] The Research Institute of the McGill University Health Centre, Montreal, QC H4A 3J1, Canada
[2] Department of Medicine, McGill University, Montreal, QC H3G 1A4, Canada
[3] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC H3G 1Y6, Canada
[4] Department of Biochemistry, McGill University, Montreal, QC H3G 1Y6, Canada

14-3-3 proteins regulate intracellular signaling pathways, such as signal transduction, protein trafficking, cell cycle, and apoptosis. In addition to the ubiquitous roles of 14-3-3 isoforms, unique tissue-specific functions are also described for each isoform. Owing to their role in regulating cell cycle, protein trafficking, and steroidogenesis, 14-3-3 proteins are prevalent in human diseases, such as cancer, neurodegeneration, and reproductive disorders, and, therefore, serve as valuable drug targets. In this review, we summarize the role of 14-3-3 proteins in normal and disease states, with a focus on 14-3-3γ and ε. We also discuss drug compounds targeting 14-3-3 proteins and their potential therapeutic uses.

Introduction

14-3-3 proteins are crucial regulators of intracellular signaling pathways. Upon interacting with their target protein, 14-3-3 proteins alter its activity, modifications, and intracellular localization [1]. The functions of 14-3-3 proteins can be categorized from two different viewpoints: isoform and tissue specificity.

Owing to their high degree of homology, researchers initially thought that 14-3-3 isoforms were redundant and, in the absence of one 14-3-3 isoform, others would compensate. Indeed, a 14-3-3γ-knockout (K/O) mouse model showed no change in brain phenotype, a finding suggesting that in brain, where 14-3-3 proteins are most abundant, another isoform replaces 14-3-3γ [2]. In-depth studies of the 14-3-3 phylogenetic tree suggest that all isoforms evolved before the divergence of mammals and that the orthologs have a higher homology compared with isoforms of the same species, implying that 14-3-3 isoforms have unique and fundamental roles [3]. This hypothesis is supported by mass-spectrometry studies identifying unique networks for each 14-3-3 isoform [4]. 14-3-3-Isoform specificity was further confirmed by studies of 14-3-3 isoform-specific K/O mice that found various tissue-specific phenotypes [5]. Based on data provided by the two main online microarray databases (http://biogps.org and http://www.proteinatlas.org), we summarize the tissue-specific expression of 14-3-3 isoforms in Fig. 1.

Certain 14-3-3 functions are similar in multiple tissues, because their target proteins are involved in global pathways. The global roles of 14-3-3 proteins can be divided into two categories: (i) cell cycle progression and apoptosis; and (ii) intracellular protein trafficking. Tissue-specific roles for 14-3-3 proteins are observed for those that target proteins in particular cell types, such as adipocytes, neurons, and testicular Leydig cells.

Functions of 14-3-3 family members

The cell cycle and apoptosis

Much work has been dedicated to understanding the role of 14-3-3 proteins in cell proliferation, growth, and apoptosis. Mitogenic signals promote proliferation through the rat sarcoma (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase (MAPK) cascade [6] and activate downstream MAP Kinase Kinase Kinase proteins (MEKK) [7]. 14-3-3ε, ζ, and θ, respectively, regulate MEKK2 dimerization in mouse embryonic fibroblast cells [8], MEKK1 functions in human prostate adenocarcinoma cell lines [9], and phosphorylation-dependent activity of MEKK3 in human fibroblasts cell lines [10]. 14-3-3 proteins are also involved in cell growth and survival. Acting through the extracellular signal-regulated kinase (ERK) pathway, 14-3-3ζ, ε, and γ mediate the activation of rapidly accelerated fibrosarcoma

Corresponding author: Papadopoulos, V. (vassilios.papadopoulos@mcgill.ca)

http://dx.doi.org/10.1016/j.drudis.2015.09.012

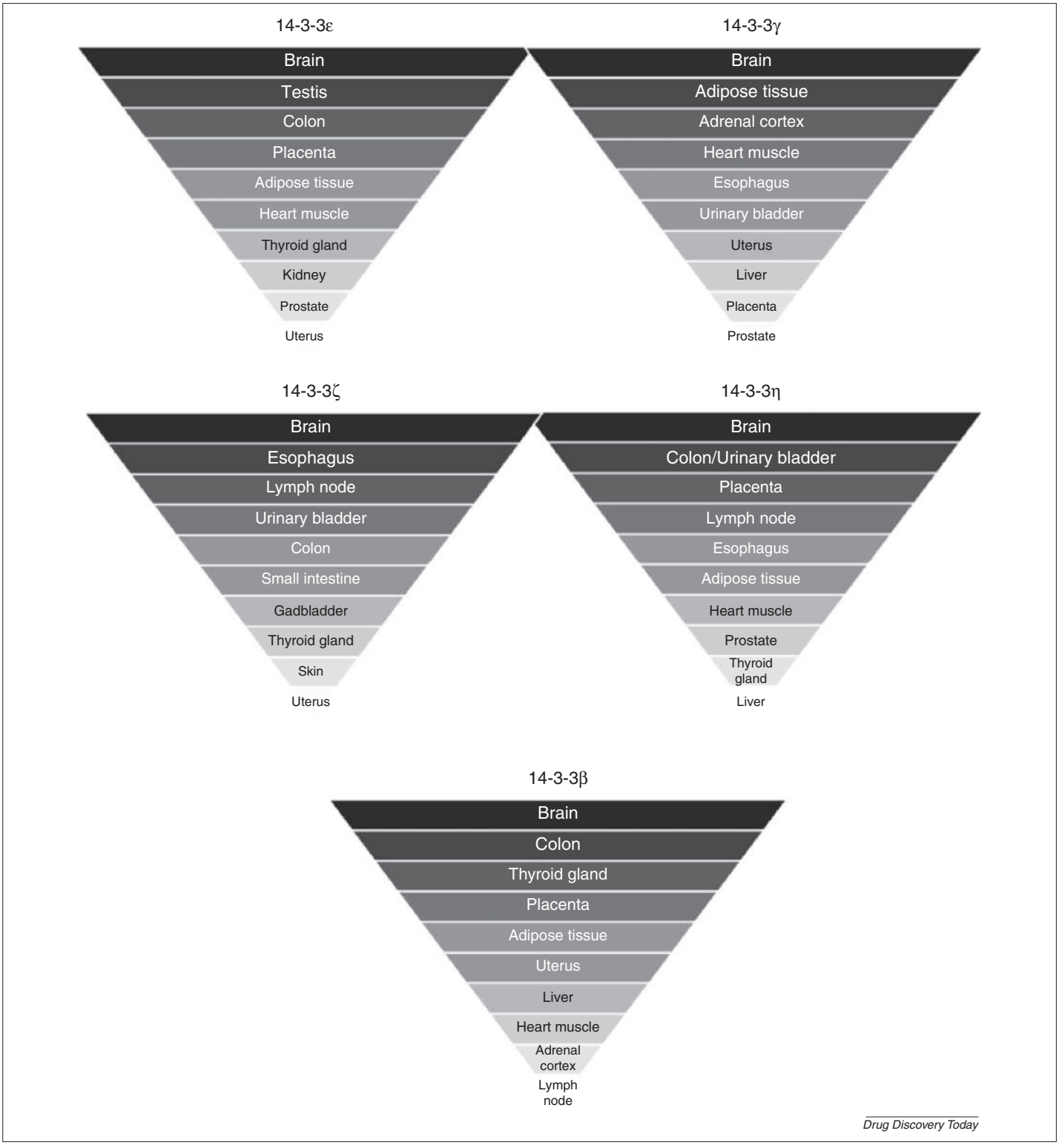

FIGURE 1

Differential expression levels of 14-3-3 isoforms in human tissues. 14-3-3 isoforms are shown in human tissues from highest to lowest expression levels as indicated by dark to light colors. Such differences suggest that there is tissue specificity for each isoform.

(Raf), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and mitogen- and stress-activated protein kinases (MSK1/2) in many cell types, including hematopoietic stem cells and human breast, prostate, and fibroblast cell lines [11–15]. Downstream of the protein kinase B (PKB or Akt) pathway, 14-3-3ζ drives cell survival by inducing the phosphorylation and deactivation of B cell lymphoma 2 (Bcl-2)-associated death promoter (BAD), which leads to inhibition of apoptosis and promotes the cytosolic retention of Forkhead (FOXO) transcription factors, thus blocking the expression of downstream pro-apoptotic genes [11] (Fig. 2a).

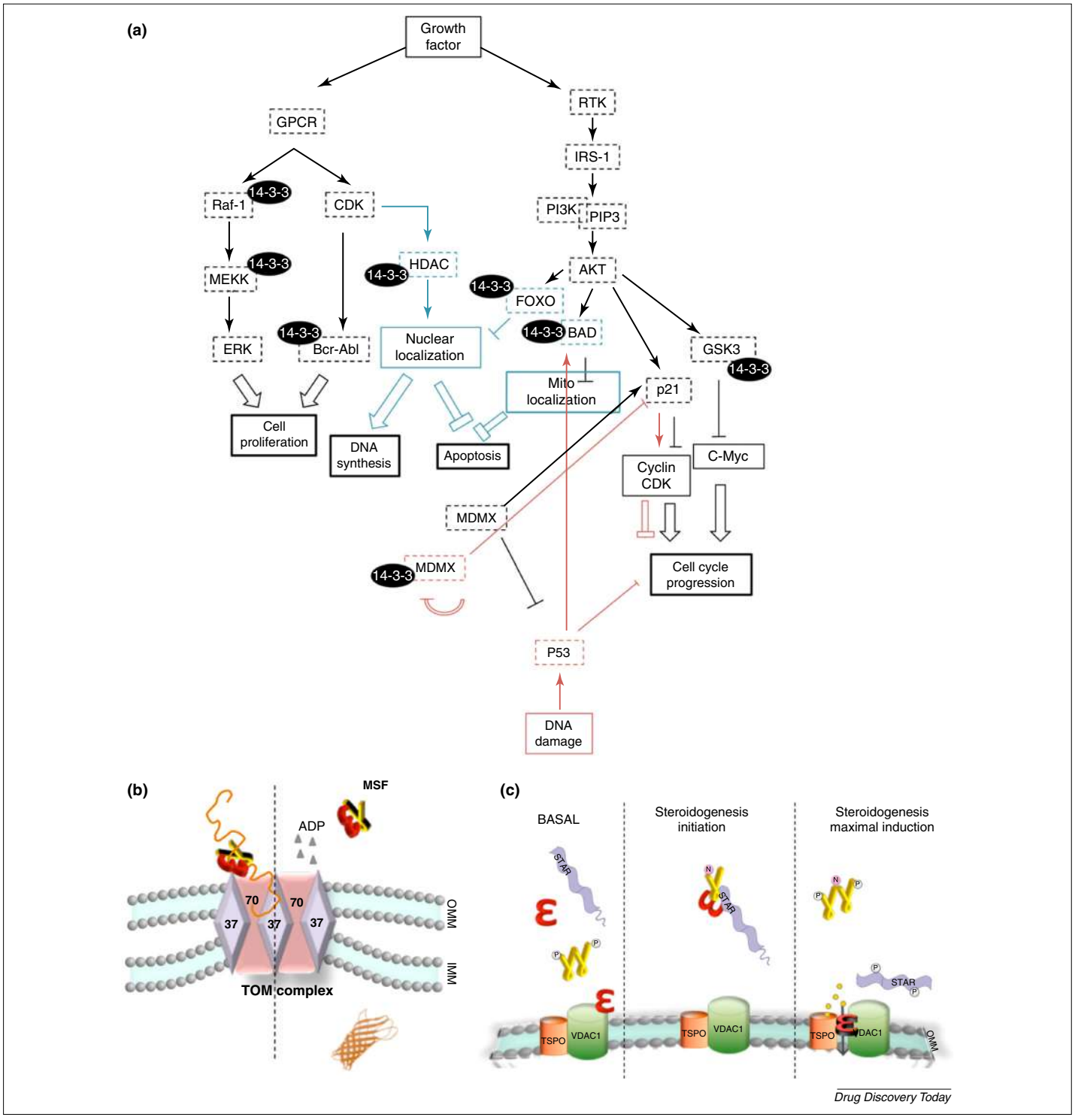

FIGURE 2

Summary of 14-3-3 protein function. **(a)** The 14-3-3 network in the cell cycle, proliferation, and apoptosis. 14-3-3 proteins are antiapoptotic and pro-proliferative, and the network of 14-3-3 protein interactions in such pathways is intricate. 14-3-3 proteins positively regulate Raf-1, MEKK proteins, and Abl proteins, modify the localization of HDAC proteins, FOXO proteins, and BAD downstream growth factors, and regulate P53 and P21 action upon DNA damage. **(b)** 14-3-3ε and γ collaborations in protein trafficking. A dimer of 14-3-3ε and γ, called mitochondrial import stimulating factor (MSF), mediates the import of β sheet-rich mitochondrial proteins through interactions with the Tom complex at the mitochondria (37, TOM subunit 37 kDa; 70, TOM subunit 70 kDa). **(c)** 14-3-3ε and γ collaborations in steroidogenesis. 14-3-3ε and γ control the rate of cholesterol import to the mitochondria by targeting STAR and VDAC1, respectively. 14-3-3γ is hormonally induced. Upon hormonal stimulation, it binds and blocks Ser194 on STAR, maintaining its basal activity. This function is transient and begins at the initiation of steroidogenesis. It is likely that 14-3-3ε is recruited by 14-3-3γ–STAR interactions. Upon 14-3-3γ release from STAR, 14-3-3ε interacts with VDAC1 at the mitochondria and buffers cholesterol import into this organelle. *Abbreviations*: AKT(PKB), protein kinase B; BAD, Bcl-2-associated dead promoter; Bcr-Abl, breakpoint cluster region protein-Abelson murine leukemia vial oncogene homolog; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; FOXO, forkhead; GPCR, G-protein-coupled receptor; GSK3, glycogen synthase kinase 3; HDAC, histone deacetylase; IMM, inner mitochondrial membrane; IRS-1, insulin receptor substrate 1; MDMX, mouse double minute X homolog; MEKK, map kinase kinase kinase protein; MITO, mitochondria; OMM, outer mitochondrial

The regulation of apoptosis by 14-3-3 proteins is a complex process. 14-3-3 proteins exert both pro- and antiapoptotic effects and, in both pathways, the main targets of 14-3-3 proteins are Bcl-2 and P53. The balance between the pro- versus antiapoptotic action of 14-3-3 isoforms was not clear until a study in 2007 provided a link between BAD and P53 regulatory loops. P53 is a tumor suppressor that is activated by cellular threats, such as DNA damage and hypoxia, and induces cell cycle arrest at G1 [16,17]. 14-3-3γ regulates P53 by blocking its inhibitors and interacting with its regulatory proteins, such as mouse double minute X homolog (MDMX) [18]. Interactions between 14-3-3γ and MDMX also block binding between MDMX and P21 (a protein required for G1 cell cycle arrest), protecting P21 from proteasomal turnover [19] (Fig. 2a).

Studies in human cell carcinoma cell lines indicate that, once the DNA damage is too extensive for repair and, therefore, G1 arrest is futile, apoptosis is triggered. P53 localizes to the nucleus and induces the transcription of genes, such as *BAD* and that encoding Bcl-2 associated X promoter (*Bax*), to begin apoptosis [20]. The BAD protein localizes to the mitochondria to induce the release of cytochrome C. 14-3-3γ, ζ, ε, β, and θ bind to phosphorylated BAD and block its entry into the mitochondria [21]. A direct interaction between 14-3-3γ and Bax is also seen in cerebral cortical neurons [22]. Through a negative feedback loop, high levels of BAD bind to P53 in the cytosol, which blocks the nuclear import of P53 and stops the transcription of *BAD* and *Bax* genes [20]. Given that such cytosolic interactions occur with nonphosphorylated BAD, a balance is maintained between 14-3-3 and P53 interactions with BAD. That is, 14-3-3 proteins are antiapoptotic when they block BAD activation but pro-apoptotic when they activate P53. This dual role is likely to be carried out by different 14-3-3 isoforms, and dimerization favors one pathway over another. 14-3-3γ and ε regulate cell division mainly through their interactions with proteins at cellular checkpoints, such as cell division cycle 25 homolog A, B and C (Cdc25A,B,C) [23].

To add another layer of complexity, 14-3-3 proteins regulate autophagy. When cells receive proper nutrition, 14-3-3 proteins interact with tuberous sclerosis 2 (TSC2), a member of the mammalian target of rapamycin (mTOR) energy sensor protein family [24]. Under starvation conditions, 14-3-3 proteins dissociate from TSC2 and interact with downstream proteins, such as Raptor and Unc51-like kinase 1 (ULK1). These processes initiate autophagy by allowing the formation of autophagosomes, which are double-membrane vesicles that degrade cellular organelles [25]. A further report also suggests a role for 14-3-3β and its phosphorylated form, 14-3-3α, in autophagy [26].

Intracellular protein trafficking

14-3-3 proteins contain a nuclear localization sequence (NLS) through which they can shuttle target proteins to the nucleus. An in-depth review by Muslin *et al.* [27] discussed such functions and therefore we discuss herein the chaperone-like activity exhibited by 14-3-3 proteins.

Part of the pathogenesis of neurodegenerative diseases is protein misfolding and subsequent aggregation; thus, 14-3-3 proteins might have a role in such disease states. Given their chaperone-like activities, some 14-3-3 isoforms can inhibit the formation of, or unravel, protein aggregates. 14-3-3 dimers and heat shock protein (Hsp)-70 anchor chaperone-associated mutant huntingtin protein aggregates to dynein, and dynein mediates the transportation of aggregates to the aggresomes [28], where proteins are degraded by autophagy. Partnerships between 14-3-3ζ and Hsp27 [29] and 14-3-3γ and Hsp20 [30] have also been reported. The pathogenesis of Parkinson's disease involves the formation of Lewy bodies, which are aggregates of β sheet-rich proteins in neurons. Co-immunoprecipitation studies identified that 14-3-3η binds α-synuclein, which is a protein abundant in Lewy bodies, and interactions between these two proteins can rescue neurons from α-synuclein aggregation and subsequent toxicity [28].

Tissue-specific functions of 14-3-3ε and γ

14-3-3ε, also known as mitochondrial import-stimulation factor (MSF) L subunit, is encoded by the *YWHAE* gene on chromosome 17 in humans. 14-3-3 phylogenetic tree analysis indicates that 14-3-3ε is the most ancient isoform of the 14-3-3 protein family, and is expressed in Plantae, Fungi, and Animalia [3]; it also has the highest degree of homology within and across species compared with other family members. 14-3-3γ is referred to as polypeptide and protein kinase C (PKC) inhibitor protein-1 (KCIP-1), if N-terminally processed. In humans, the *YWHAG* gene encoding 14-3-3γ is located on chromosome 7. 14-3-3γ and ε are highly expressed in mammalian brain and have a high affinity for heterodimerization with one another [31]; therefore, several unique interactions between the two proteins are found in the regulation of tissue-specific pathways.

Mitochondrial protein import

The heterodimer of 14-3-3ε with γ or ζ was previously named mitochondrial import stimulating factor (MSF) [32]. Studies in rat liver extracts indicated that MSF is a unique chaperone that recognizes the N-terminal mitochondria sequences on matrix-targeted proteins in the cytosol. MSF maintains the unfolded, import-competent state of preproteins, and unfolds misfolded preproteins. High concentrations of ADP at the mitochondrial membrane can cause MSF–target dissociation. An example of MSF-mediated protein import has been shown for the mitochondrial matrix protein preadrenodoxin, a part of the preprotein assembly and machinery (PAM) complex that assists in the assembly of cytochrome P450 side-chain cleavage (CYP11A1) in the inner mitochondrial membrane [33]. MSF forms a transient bond with translocase of outer mitochondria (Tom) isoforms 70 and 37 (Tom70–Tom37), which are the analogs of sorting and assembly machinery (SAM) protein complexes in yeast. SAM complexes mediate the import of β-barrel proteins to the mitochondrial matrix [34] (Fig. 2b).

membrane; PI3K, phosphatidylinositol-4,5 bisphosphate 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; Raf-1, rapidly accelerated fibro sarcoma 1; RTK, receptor tyrosine kinase; STAR, steroidogenic acute regulatory protein; TOM, translocase of the outer mitochondria; TSPO, translocator protein; VDAC1, voltage-dependent anion channel 1.

TABLE 1

Summary of 14-3-3 isoform-specific K/O phenotypes in mice

K/O isoform	Tissue studied	Phenotype	Refs
14-3-3γ	Brain	No phenotype	[2]
14-3-3ε	Brain	Schizophrenic behavior, impaired memory	[65]
14-3-3ζ	Brain	Schizophrenic behavior, impaired learning and memory, reduced prepulse inhibition, locomotor hyperactivity	[66,67]
14-3-3ε and ζ	Brain	Defects in proliferation and differentiation of neural progenitor cells in the cortex, neuronal migration defects, and seizures	[68]

Interactions with cellular compartments

14-3-3γ and ε are the only isoforms that bind to phospholipid aggregates in vesicles and membrane proteins [35]. Studies using surface plasma resonance identified His158 and 195 (located in the amphipathic helices at the N-terminal dimerization region of the 14-3-3γ isoform) as the optimal residues for interaction with membranes [36]. In humans, Trp60 on 14-3-3γ binds to negatively charged membranes [37], and 14-3-3γ interacts with cytoskeletal actin filaments and contributes to the regulation of apoptosis [38]. 14-3-3γ also interacts with organelle membrane of lysosomes, mitochondria, and Golgi. In a groundbreaking study, Mityamoto *et al.* showed that 14-3-3γ is enriched at the mitochondria during lysosomal accumulation. Loss of this isoform inhibited the elimination of oxidized mitochondrial proteins, suggesting that it has a role in mitochondrial quality control [39]. At the Golgi apparatus, 14-3-3γ interacts with large pleomorphic carriers that travel from the Golgi complex to the plasma membrane and undergo fission. Disrupting such interactions inhibits carrier budding and fission processes [40].

Steroidogenesis regulation

Cholesterol import into the mitochondria is the rate-limiting step in steroidogenesis. It is hormone dependent and mediated through a protein complex called the transduceosome. This complex contains proteins such as steroidogenic acute regulatory protein (STAR), translocator protein (TSPO), and voltage-dependent anion channel 1 (VDAC1) [41]. 14-3-3ε and γ interact with STAR, TSPO, and VDAC1, and control the rate of cholesterol import [42,43]. Two distinct but complementary mechanisms of action have been identified for 14-3-3γ and ε. Hormone treatment increases the levels of 14-3-3γ and alters its post-translational modifications (PTMs), leading to the disruption of 14-3-3γ homodimers [44]. Consequently, 14-3-3γ monomers interact with STAR on Ser194 within the 14-3-3 binding motif, which physically blocks the accessibility of this residue for protein kinase A (PKA) phosphorylation. This phosphorylation is required for the twofold induction of STAR activity and maximal steroidogenesis [44]. 14-3-3γ–STAR interactions are short-lived and delay the initiation of steroidogenesis because 14-3-3γ quickly dissociates from STAR due to PTM-driven homodimerization. This delay in steroidogenesis might be required to prepare the steroidogenic machinery and make sufficient cholesterol substrate available, but the details remain unknown.

14-3-3ε regulates long-term steroidogenesis. Cytosolic STAR and mitochondrial VDAC1 compete for binding with 14-3-3ε. 14-3-3γ–STAR interactions assist in recruiting 14-3-3ε to the proximal 14-3-3 binding motif of STAR that contains Ser187. Consequently, 14-3-3ε–VDAC1 interactions at the mitochondria are decreased. Upon the release of 14-3-3γ from STAR, VDAC1 can compete with STAR for binding with 14-3-3ε, meaning that the interactions between VDAC1 and 14-3-3ε are not only restored, but also significantly increased [43]. 14-3-3ε interacts with VDAC1 on the Ser167 residue of the channel. This binding results in multiple downstream effects, including localization of 14-3-3ε to the mitochondria, negative regulation of steroidogenesis by buffering cholesterol import through intercalation between TSPO and VDAC1, and regulation of cholesterol and ligand binding to TSPO. The negative regulation of 14-3-3ε in testosterone production has been shown *in vivo* in adult rats, and the interactions with VDAC1 were confirmed in testes sections [43] (Fig. 2c).

14-3-3 proteins in disease and treatment

Neurological disorders and neurodegeneration

14-3-3 proteins exert neuroprotective properties and are known markers of several neural diseases. Here, we discuss some of the studies of the relevance of 14-3-3 proteins in neurological diseases.

14-3-3ε targets NudE Neurodevelopment Protein 1-Like 1 (Ndel1) and lissencephaly-1 (Lis1), thus regulating neural migration by recruiting microtubules and assisting their movement [5]. 14-3-3ε heterozygous K/O mice display alterations in hippocampal and cortical structures and exhibit behavioral phenotypes, such as memory loss [5]. These K/O animals are used as schizophrenia-related models (Table 1). In 2003, Toyo-oka *et al.* showed that heterozygous deletion of 17p13.3 genes, including 14-3-3ε, causes Miller–Dieker syndrome, a form of lissencephaly (or 'smooth brain' disease) caused by a lack of neural migration. Microdeletions in the 14-3-3ε gene manifest in patients as autistic characteristics, facial dysmorphic features, and postnatal overgrowth [45]. The correlations between lack of 14-3-3γ, ζ, and ε with memory acquisition impairment, astrocyte apoptosis, schizophrenic-type behavior, Parkinson's disease, Alzheimer disease, and bipolar disorder are reviewed thoroughly by Foote *et al.* [5]. The importance of 14-3-3 isoform-specific functions in the brain is well studied in 14-3-3 isoform-specific K/O mouse models, as summarized in Table 1.

In a series of interesting studies, ubiquitin-derived proteasomal degradation produced functional oligopeptides that could target proteins and regulate cell signaling [46]. Two of these peptides isolated from mouse [47] or rat brain targeted recombinant 14-3-3ε and blocked its interactions with target proteins, such as calmodulin kinase (CaM kinase). As a consequence, the Ca^{2+} levels in the cell increased, serving as a second messenger for a variety of signal transduction pathways. Some researchers suggest that these oligopeptides endogenously bind to proteins and block their protein-binding domains. These interactions can be ablated by

TABLE 2

Summary of compounds that can be used as potential therapeutics through targeting 14-3-3 proteins

Name	Compound	Function	Proposed application	Host cell	Refs
Compound 19	Peptidomimetic	Induces nuclear localization of FOXO3A	Cancer therapy	Human DG-75 lymphoma cells	[53]
Difopein (R18 dimer)	Synthetic peptide	Displaces 14-3-3–BAD interactions	Cancer therapy	Mouse embryonic fibroblast (NIH 3T3)	[21]
FC	Diterpene glucoside	Blocks ERα dimerization	Cancer therapy	Human ovarian, lung, prostate, and colon carcinoma cells	[55]
FC-THF	Diterpene glucoside	Blocks ER retention	Cancer therapy	*Xenopus* oocytes	[57]
Cotylenin A	Peptide	Stabilizes 14-3-3c-RAF interactions	Cancer therapy	Epidermoid and rectal carcinoma	[58]
Molecular tweezers	Norbornadiene and benzene rings	Displaces 14-3-3-ph.C–Raf and 14-3-3–Exo S	Cancer therapy	–	[59]
FOBISIN 101	Pyridoxal-phosphate moiety linked to p-amino-benzoate	Displaces 14-3-3–Raf-1 interactions	Cancer therapy	Human lung cancer cells	[60]
UTKO1	Moverastin A derivative	Displaces 14-3-3–Raf-1 interactions	Cancer therapy	Human epidermoid carcinoma cells	[62]
TVS167	Peptide	Displaces 14-3-3ε–VDAC1 interactions	Hypogonadism	Mouse MA-10 Leydig cells; rat Leydig cells; *in vivo* in Sprague-Dawley rats	[43]

phosphorylation, which suggests that 14-3-3ε is regulated by these endogenously produced natural peptides. It might prove beneficial to utilize these peptides as drug substitutes to target 14-3-3ε in neurodegenerative diseases. Such studies provide a novel therapeutic area in 14-3-3 drug development.

Cancer

14-3-3 proteins target oncogenic proteins, as previously discussed. High expression of 14-3-3ζ is associated with lung, breast, prostate, myeloma, glioma, esophageal, head and neck, oral, pancreatic, ovarian, and skin cancers. These elevated expression levels correlate with poor prognosis and chemoresistance for some, but not all, of these cancer types [48]. Small interfering RNA (siRNA) knockdown (K/D) of 14-3-3ζ inhibits cancer cell growth progression [48], highlighting the potential of 14-3-3ζ as a pharmaceutical drug target. 14-3-3γ and ε are novel markers of hepatocellular carcinoma [48], low levels of 14-3-3γ and θ are markers of breast cancer [23,49], and high levels of 14-3-3β serve as a marker for gastric cancer [50]. siRNA K/D of 14-3-3β arrests tumorigenesis and astrocytoma progression, making this protein a potential therapeutic candidate.

By contrast, the more distant family member 14-3-3σ is a prominent tumor suppressor. Low levels of 14-3-3σ are used to determine the prognosis of cancer in tissues with mesenchymal origin [51]. More thorough studies indicate that low levels of 14-3-3σ result from either ubiquitin-derived proteasomal degradation or hypermethylation of the 14-3-3σ promoter, which leads to gene silencing and results in breast cancer progression. Therefore, 14-3-3σ methylation can be used as a diagnostic marker [51].

With the acknowledgement of 14-3-3 proteins as potential therapeutic targets, there is an increased effort to pharmacologically target these proteins in cancer.

In a recent study, a prodrug peptidomimetic called compound 19 (C19) was identified in a small-molecule microassay. This compound was synthesized and modified to improve its characteristics, creating compound 15 (C15). Treatment of DG75 leukemia cells with C15 showed that this compound drives apoptosis in a dose-sensitive manner by activating FOXO3A-derived proapoptotic gene transcription. Further investigations showed that, once inside the cell, C15 is converted to C19a, a phosphoserine mimetic prodrug. Active C19a can interact with 14-3-3 proteins, such as 14-3-3θ, with an IC_{50} of 5 μM, and block their interactions with FOXO3A. As previously noted, 14-3-3 proteins target phosphorylated FOXO3A downstream of the Akt pathway and thereby block nuclear localization and subsequent gene transcription. Thus, C19a drives apoptosis by blocking these interactions and allowing FOXO3A to enter the nucleus. C19a has been proposed as a candidate for cancer treatment; however, because this phosphoserine mimetic produrg is not pathway specific, it could block the interaction of 14-3-3 proteins with other targets. Given that 14-3-3 proteins exert global functions in most mammalian tissues, the cancer tissue specificity of the compound could be questioned and limit its potential clinical use [52] (Fig. 3a).

Another 14-3-3 targeting compound is the synthetic peptide PHCVPRDLSWLDLEANMCLP, named R18, identified from a phage display library. The core amino acids WLDLE occupy the amphipathic groove of 14-3-3 proteins, and the R18 sequences are not phosphorylated. Electron density maps calculated for recombinant 14-3-3ζ crystals in complex with Raf-1 kinase to R18 indicate that the two peptides bind to different sites with similar affinity. Moreover, both peptides interact with 20 residues in the 14-3-3 amphipathic groove, some of which overlap (e.g. Lys49 and Arg56). This explains the mechanism by which R18

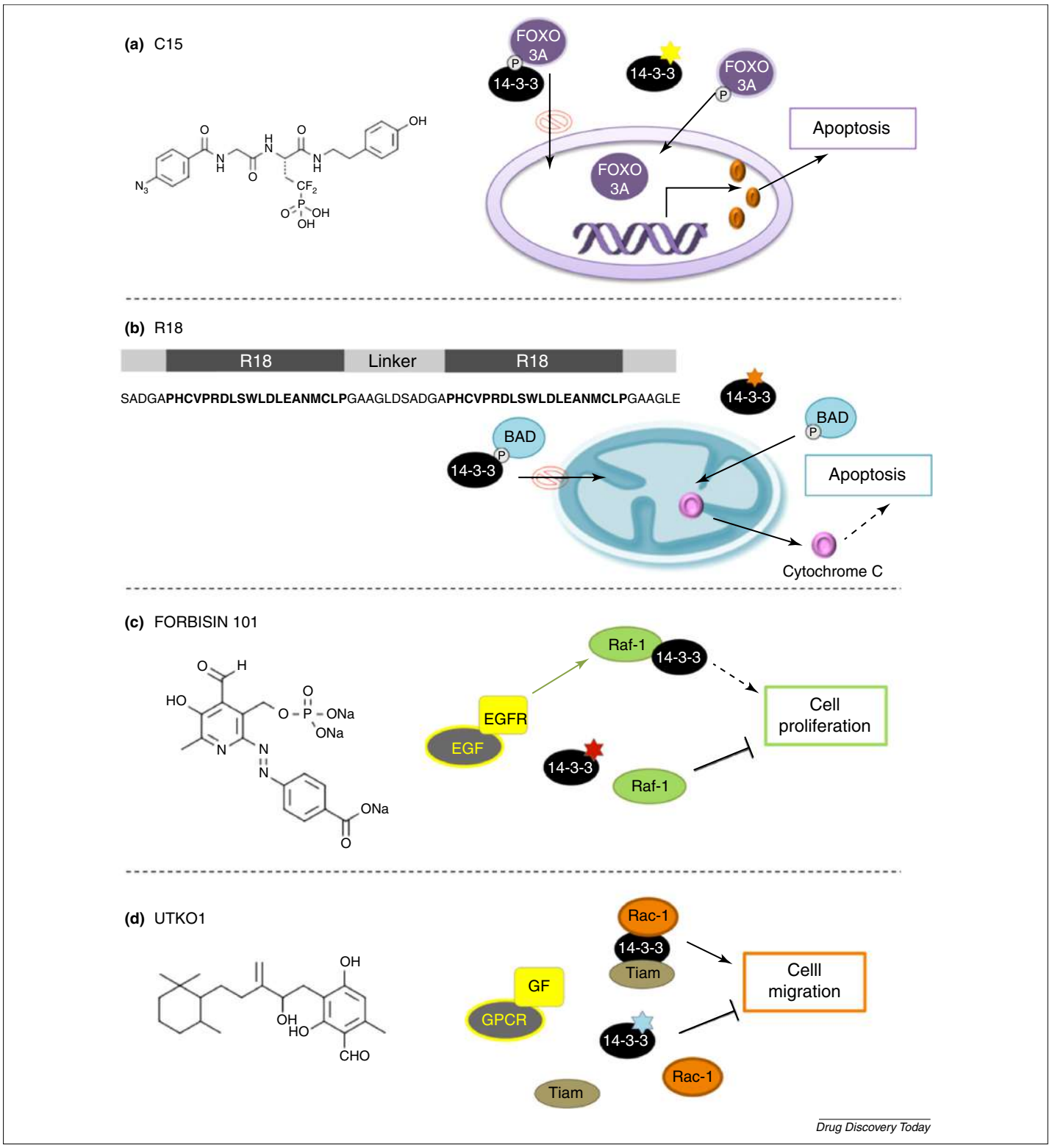

FIGURE 3

Drug compounds that block 14-3-3-target interactions. **(a)** Compound 19a (yellow star) is a phospho-peptidomimetic prodrug that targets the 14-3-3 amphipathic groove. Under normal conditions, 14-3-3 proteins interact with phosphorylated FOXO3A and retain this transcription factor in the cytosol. Compound 19a blocks 14-3-3-FOXO3a interactions, causing FOXO3A localization to the nucleus and the subsequent transcription of pro-apoptotic genes. **(b)** Difopein (orange star) is a dimer of R18, a 14-3-3-targeting short peptide. Treating cells with an R18 dimer displaces 14-3-3 proteins from BAD by occupying the 14-3-3 target-binding pocket. BAD then localizes to the mitochondria and induces the release of cytochrome C, causing apoptosis. **(c)** FOBISIN 101 (red star) targets 14-3-3 proteins and blocks the interactions between 14-3-3 and Raf-1, a kinase downstream of the EGF signaling pathway, therefore blocking Raf-1-induction of cell proliferation. **(d)** UTKO1 (purple star) is a moverastin derivative that binds to 14-3-3 proteins cells and blocks 14-3-3 interactions with its targets, for example, Rac-1. Rac-1 is activated when bridged to Tiam by 14-3-3 proteins. In the presence of UTKO1, 14-3-3 proteins can no longer adapt Tiam–Rac-1 interactions and, therefore, cell migration is blocked. *Abbreviations*: BAD, Bcl-2-associated dead promoter; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FOXO, forkhead; GF, growth factor; GPCR, G-protein-coupled receptor; Rac-1, Ras-related C3 botulinum toxin substrate 1; Raf-1, rapidly accelerated fibro sarcoma 1; Tiam, T-lymphoma invasion and metastasis-inducing protein.

binding blocks Raf-1 kinase interactions with 14-3-3ζ. In this study, the physiological outcome was not evaluated [53]. A dimeric 14-3-3 peptide inhibitor (Difopein) was developed that comprised two R18 sequences attached by a linker sequence. This peptide inhibitor binds with high affinity to 14-3-3ζ, displaces 14-3-3–BAD interactions (K_d = 80 nM) in NIH 3T3 cells and, thus, results in apoptosis [21] (Fig. 3b).

Some of the identified 14-3-3 targeting compounds are found in nature. The diterpene glucoside fusicoccin (FC), isolated from the fungus *Phomopsis amygdali*, a liverwort, plants, and insects [54], was shown to induce apoptosis in several human cancer cell lines, including ovarian, lung, prostate, and colon carcinoma cells [55]. FC was found to stabilize the interactions between 14-3-3 proteins and estrogen receptor α (ERα) in breast cancer cells. These interactions mask the dimerization domain of ERα, which is required for chromatin interactions and downstream gene expression. Thus, by stabilizing the 14-3-3–ERα interactions, FC blocks breast cancer cell proliferation. Co-crystallization of the ERα in complex with 14-3-3 proteins and FC indicated that the 14-3-3–ERα interactions occur with high affinity upon phosphorylation of Thr594 on ERα and inhibit the estrogen-driven dimerization of the receptor [54].

A semisynthetic FC derivative, FC-THF, was found to stabilize the interactions between 14-3-3 proteins and the plasma membrane K^+ channels TWIK-related acid-sensitive K^+ channels 1 and 3 (TASK-1 and TASK-3). Studies in *Xenopus laevis* oocytes indicated that this interaction results from the presence of 14-3-3 mode 3 motif on TASK1/3 proteins, through which 14-3-3 proteins block the endoplasmic reticulum retention sequences, thus favoring the localization of TASK1/3 to the plasma membrane [56]. TASK proteins are upregulated in breast, lung, colon, and metastatic prostate cancers, and enhancement of the K^+ current is directly involved in apoptosis and oncogenesis [57], highlighting the potential of FC-THF in cancer treatment.

A similar mechanism of action to FC was reported for Cotylenin A, a bioactive fungal metabolite that shows anticancer properties in some human cancers. Cotylenin A exhibits antitumor activity in A431 and Difi cancer cells through targeting 14-3-3 proteins and stabilizing their interactions with oncogenic targets, such as C-RAF [58]. 14-3-3 proteins interact with the phosphorylated Ser233 and Ser259 on C-RAF with an EC_{50} of 65.44 μM. C-RAF mutations are common in human cancers and Cotylenin A was found to be inactive in cancer lines expressing mutated C-RAF [58]. Moreover, water-soluble molecular tweezers were recently identified to block the interactions between 14-3-3σ and C-RAF phosphorylated on S259 [59].

A covalent 14-3-3 inhibitor called FOBISIN 101 was recently identified from the Library of Pharmacologically Active Compounds (LOPAC). This compound is a pyridoxal-phosphate moiety linked by a nitrogen double bound to p-amino-benzoate. FOBISIN 101 is a pan-14-3-3 inhibitor that binds to the 14-3-3-conserved Lys120 residue in the proximity of the amphipathic groove with an IC_{50} of 2.6 μM in lung cancer cells and thereby inhibits the binding of phosphorylated targets [e.g. Raf-1 and proline-rich Akt substrate of 40 kDa (PRAS40)] and nonphosphorylated targets (e.g. ExoS) in a dose-dependent manner [60]. The hypothesized downstream cellular consequence of using FOBISIN 101 is presented in Fig. 3c.

Previous research demonstrated that a member of the cylindrol family of microbial origin, called moverastin, blocks cancer metastasis by inhibiting cell migration. *In vitro*, moverastin targets farnesyl transferases (FTases) and blocks their function, which is followed by membrane localization of H-Ras, downstream of the PI3K/Akt pathway in human esophageal tumor cells [61]. Interestingly, a chemically synthesized moverastin derivative called UTKO1 was more potent than moverastin but did not show any correlation with FTases [62]. Further studies indicate that epidermal growth factor (EGF) signaling activates Rac-1, which is involved in cell migration, by triggering the formation of the ternary complex of Ras-related C3 botulinum toxin substrate 1 (Rac-1)/14-3-3ζ/T-lymphoma invasion and metastasis-inducing protein

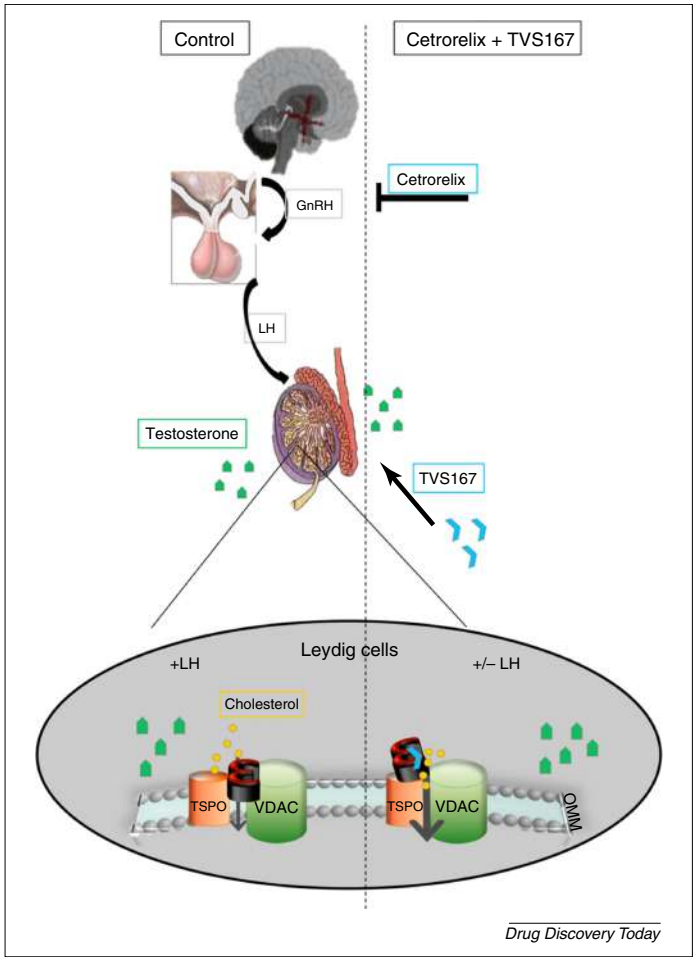

FIGURE 4

TVS167 blocks 14-3-3ε-VDAC1 interactions in Leydig cells and thereby induces testosterone production. Testosterone production is triggered when LH is produced by the pituitary gland in response to GnRH. LH interacts with its receptor on Leydig cells of the testis and triggers the movement of cholesterol from the cytosol to the mitochondria through interactions with VDAC1 and TSPO. To tightly regulate this import rate, 14-3-3ε intercalates between these two proteins and blocks efficient interactions. TVS167 is a short peptide containing VDAC1 Ser167 in a 14-3-3 motif that competes with VDAC1 to interact with 14-3-3ε. This blockade induces the flow of cholesterol to the mitochondria and testosterone production. If LH signaling is blocked using cetrorelix, the GnRH antagonist TVS167 can be utilized to drive testosterone production. *Abbreviations*: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; OMM, outer mitochondrial membrane; TSPO, translocator protein; VDAC1, voltage-dependent anion channel 1.

(Tiam). Co-immunoprecipitation studies using UKTO1-coated beads in human epidermoid carcinoma cells showed that this compound targets 14-3-3ζ, blocking its interactions with Rac-1, with an IC_{50} of 1.98 μM, thereby inhibiting protein activation and subsequent cancer cell migration [63] (Fig. 3d).

Taken together, these data indicate that 14-3-3 proteins are potential drug targets for cancer treatment. However, the compounds identified (Table 2) are not specific and could also affect 14-3-3 interactions with other targets. Therefore, these compounds are not currently viewed as selective for a particular pathway.

Hypogonadism

Reduced serum testosterone occurs commonly in 20–50% of aging men more than 60 years old and in subfertile or infertile young men. Low levels of testosterone are linked to several diseases, including infertility, cardiovascular diseases, adrenal or testicular hyperplasia, neurodegeneration, altered mood, fatigue, decreased lean body mass and bone mineral density, increased visceral fat, metabolic syndrome, and decreased libido and sexual function. Therefore, clinical methods, such as testosterone replacement therapy (TRT), have been developed to address such symptoms [64].

Given that adverse effects of TRT render this treatment unsuitable for many patients [64], alternate mechanisms to elevate testosterone levels are desirable. 14-3-3ε, a negative regulator of testosterone production, was successfully targeted in rat models to induce endogenous testosterone production in Leydig cells of testes. A peptide, called TVS167, was created that competes with VDAC1 for binding to 14-3-3ε, thus removing the negative regulation of 14-3-3ε on VDAC1 in rat testes, leading to increased testosterone levels in intratesticular fluid and serum in a dose-dependent manner. Moreover, blocking these interactions increased testosterone production in a hypogonadal rat model [43], indicating that this use is applicable for patients with limited luteinizing hormone (LH) production or signaling defects (Fig. 4). Further studies indicate that the capacity of the testes to regulate intratesticular testosterone release was not jeopardized by TVS167, suggesting that this compound offers a promising alternative to TRT. Moreover, this peptide is specific for testes rather than for other steroidogenic tissues, such as adrenals. Given the high sequence homology and similar tertiary structures between VDAC1 and 14-3-3ε in rat and human species, TVS167 might be a promising tool for the treatment of hypogonadal patients. Noteworthy, unlike the 14-3-3-targeting drugs discussed in cancer treatment, TVS167 specifically blocks VDAC1 interactions while allowing 14-3-3ε to function in other cellular processes (Table 2). Therefore, TVS167 does not compromise cell viability.

Concluding remarks

14-3-3 family members regulate the cell cycle and apoptosis globally in mammalian tissues by targeting proteins, such as P53 and BAD, and regulating processes, such as autophagy [18,19]. Therefore, their value in pharmaceutical drug development has been long recognized. The lack of 14-3-3 isoform, tissue, and target specificity for currently available compounds raises concerns because induction of apoptosis might occur in healthy tissues as an adverse effect. 14-3-3 family members also assist in protein trafficking by altering target protein localization and chaperone-like activity. Hence, unregulated 14-3-3 expression levels, mutations, epigenetic factors, and targeted interactions might lead to a range of neurodegenerative diseases. Recent studies have introduced natural peptides that target 14-3-3ε in the brain and that could be used as potential treatments for early disease states [47]. Other studies have shed light on novel peptides as drug candidates that abrogate 14-3-3ε–target interactions with tissue and target specificity in the testis, promising therapies for restoring testosterone production in hypogonadal men [43]. Thus, the availability of more specific biologics targeting 14-3-3 protein interactions could open the door for the use of these molecular targets in cancer, neurodegeneration, and reproductive disease treatments.

Conflict of interest

The authors are named inventors in a patent (US 61/953,336; PCT/CA2014/050467) filed by McGill University.

Acknowledgments

The authors were supported by grants from the Canadian Institutes of Health Research (MOP102647 and 125983) and a Canada Research Chair in Biochemical Pharmacology to V.P. and fellowships from the Research Institute of MUHC and the McGill Center for the Study of Reproduction to Y.A.

References

1 Tzivion, G. *et al.* (2001) 14-3-3 proteins; bringing new definitions to scaffolding. *Oncogene* 20, 6331–6338
2 Steinacker, P. *et al.* (2005) Unchanged survival rates of 14-3-3gamma knockout mice after inoculation with pathological prion protein. *Mol. Cell Biol.* 25, 1339–1346
3 Wang, W. and Shakes, D.C. (1996) Molecular evolution of the 14-3-3 protein family. *J. Mol. Evol.* 43, 384–398
4 Uhart, M. and Bustos, D.M. (2013) Human 14-3-3 paralogs differences uncovered by cross-talk of phosphorylation and lysine acetylation. *PLOS ONE* 8, e55703
5 Foote, M. and Zhou, Y. (2012) 14-3-3 proteins in neurological disorders. *Int. J. Biochem. Mol. Biol.* 3, 152–164
6 Campbell, S.L. *et al.* (1998) Increasing complexity of Ras signaling. *Oncogene* 17, 1395–1413
7 Guan, K.L. (1994) The mitogen activated protein kinase signal transduction pathway: from the cell surface to the nucleus. *Cell Signal.* 6, 581–589
8 Matitau, A.E. *et al.* (2013) MEKK2 kinase association with 14-3-3 protein regulates activation of c-Jun N-terminal kinase. *J. Biol. Chem.* 288, 28293–28302
9 Sun, B.K. *et al.* (2011) MEKK1/MEKK4 are responsible for TRAIL-induced JNK/p38 phosphorylation. *Oncol. Rep.* 25, 537–544
10 Adams, D.G. *et al.* (2002) Phosphorylation of the stress-activated protein kinase, MEKK3, at serine 166. *Arch. Biochem. Biophys.* 407, 103–116
11 Thomas, D. *et al.* (2005) 14-3-3 protein signaling in development and growth factor responses. *Curr. Top. Dev. Biol.* 67, 285–303
12 Khan, P. *et al.* (2013) Mitogen-and stress-activated protein kinases 1 and 2 are required for maximal trefoil factor 1 induction. *PLOS ONE* 8, e63189
13 Shukla, S. *et al.* (2014) Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoXO pathway. *Carcinogenesis* 452–460
14 Radhakrishnan, V.M. and Martinez, J.D. (2010) 14-3-3gamma induces oncogenic transformation by stimulating MAP kinase and PI3 K signaling. *PLoS ONE* 5, e11433
15 Radhakrishnan, V.M. *et al.* (2012) Activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling and the consequent induction of transformation by overexpressed 14-3-3gamma protein require specific amino acids within 14-3-3gamma N-terminal variable region II. *J. Biol. Chem.* 287, 43300–43311

16 Graeber, T.G. *et al.* (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 379, 88–91
17 Jones, A. (1998) A general review of the p53 gene and oral squamous cell carcinoma. *Ann. R. Australas. Coll. Dent. Surg.* 14, 66–69
18 Jin, Y. *et al.* (2006) 14-3-3gamma binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation. *EMBO J.* 25, 1207–1218
19 Lee, J.H. and Lu, H. (2011) 14-3-3Gamma inhibition of MDMX-mediated p21 turnover independent of p53. *J. Biol. Chem.* 286, 5136–5142
20 Jiang, P. *et al.* (2007) p53 and Bad: remote strangers become close friends. *Cell Res.* 17, 283–285
21 Masters, S.C. *et al.* (2002) Survival-promoting functions of 14-3-3 proteins. *Biochem. Soc. Trans.* 30, 360–365
22 Lai, X.J. *et al.* (2014) Selective 14-3-3gamma induction quenches p-beta-catenin Ser37/Bax-enhanced cell death in cerebral cortical neurons during ischemia. *Cell Death Dis.* 5, e1184
23 Mhawech, P. (2005) 14-3-3 proteins: an update. *Cell Res.* 15, 228–236
24 Tokunaga, C. *et al.* (2004) mTOR integrates amino acid- and energy-sensing pathways. *Biochem. Biophys. Res. Commun.* 313, 443–446
25 Pozuelo-Rubio, M. (2012) 14-3-3 proteins are regulators of autophagy. *Cells* 1, 754–773
26 Clapp, C. *et al.* (2012) 14-3-3 protects against stress-induced apoptosis. *Cell Death Dis.* 3, e348
27 Muslin, A.J. and Xing, H. (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.* 12, 703–709
28 Jia, B. *et al.* (2014) 14-3-3 and aggresome formation: implications in neurodegenerative diseases. *Prion* 8, 28123
29 Zhao, G.Y. *et al.* (2014) The overexpression of 14-3-3zeta and Hsp27 promotes non-small cell lung cancer progression. *Cancer* 120, 652–663
30 Chernik, I.S. *et al.* (2007) Small heat shock protein Hsp20 (HspB6) as a partner of 14-3-3gamma. *Mol. Cell. Biochem.* 295, 9–17
31 Aitken, A. *et al.* (2002) Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem. Soc. Trans.* 30, 351–360
32 Aitken, A. (2006) 14-3-3 proteins: a historic overview. *Semin. Cancer Biol.* 16, 162–172
33 Hachiya, N. *et al.* (1995) Reconstitution of the initial steps of mitochondrial protein import. *Nature* 705–709
34 Suzuki, H. *et al.* (2002) Characterization of rat TOM70 as a receptor of the preprotein translocase of the mitochondrial outer membrane. *J. Cell Sci.* 115, 1895–1905
35 Roth, D. *et al.* (1994) Characterization of 14-3-3 proteins in adrenal chromaffin cells and demonstration of isoform-specific phospholipid binding. *Biochem. J.* 301, 305–310
36 Bustad, H.J. *et al.* (2012) The peripheral binding of 14-3-3gamma to membranes involves isoform-specific histidine residues. *PLoS ONE* 7, e49671
37 Bustad, H.J. *et al.* (2011) The binding of 14-3-3gamma to membranes studied by intrinsic fluorescence spectroscopy. *FEBS Lett.* 585, 1163–1168
38 Chen, X.Q. and Yu, A.C. (2002) The association of 14-3-3gamma and actin plays a role in cell division and apoptosis in astrocytes. *Biochem. Biophys. Res. Commun.* 296, 657–663
39 Miyamoto, T. *et al.* (2012) Identification of 14-3-3gamma as a Mieap-interacting protein and its role in mitochondrial quality control. *Sci. Rep.* 2, 379
40 Valente, C. *et al.* (2012) A 14-3-3gamma dimer-based scaffold bridges CtBP1-S/BARS to PI(4)KIIIbeta to regulate post-Golgi carrier formation. *Nat. Cell Biol.* 14, 343–354
41 Aghazadeh, Y. *et al.* (2015) Pharmacological regulation of the cholesterol transport machinery in steroidogenic cells of the testis. *Vitam. Horm.* 98, 189–227
42 Aghazadeh, Y. *et al.* (2012) Hormone-induced 14-3-3gamma adaptor protein regulates steroidogenic acute regulatory protein activity and steroid biosynthesis in MA-10 Leydig cells. *J. Biol. Chem.* 287, 15380–15394
43 Aghazadeh, Y. *et al.* (2014) Induction of androgen formation in the male by a TAT-VDAC1 fusion peptide blocking 14-3-3varepsilon protein adaptor and mitochondrial VDAC1 interactions. *Mol. Ther.* 22, 1779–1791
44 Aghazadeh, Y. *et al.* (2014) Protein modifications regulate the role of 14-3-3gamma adaptor protein in cAMP-induced steroidogenesis in MA-10 Leydig cells. *J. Biol. Chem.* 289, 26542–26553
45 Toyo-oka, K. *et al.* (2003) 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller-Dieker syndrome. *Nat. Genet.* 34, 274–285
46 Cunha, F.M. *et al.* (2008) Intracellular peptides as natural regulators of cell signaling. *J. Biol. Chem.* 283, 24448–24459
47 Russo, L.C. *et al.* (2012) Natural intracellular peptides can modulate the interactions of mouse brain proteins and thimet oligopeptidase with 14-3-3epsilon and calmodulin. *Proteomics* 12, 2641–2655
48 Tzivion, G. *et al.* (2006) 14-3-3 proteins as potential oncogenes. *Semin. Cancer Biol.* 16, 203–213
49 Song, Y. *et al.* (2012) Expression of 14-3-3gamma in patients with breast cancer: correlation with clinicopathological features and prognosis. *Cancer Epidemiol.* 36, 533–536
50 Ma, Y. *et al.* (2014) Identification of proteins associated with lymph node metastasis of gastric cancer. *J. Cancer Res. Clin. Oncol.* 140, 1739–1749
51 Li, Z. *et al.* (2009) 14-3-3sigma, the double-edged sword of human cancers. *Am. J. Transl. Res.* 1, 326–340
52 Arrendale, A. *et al.* (2012) Synthesis of a phosphoserine mimetic prodrug with potent 14-3-3 protein inhibitory activity. *Chem. Biol.* 19, 764–771
53 Petosa, C. *et al.* (1998) 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. *J. Biol. Chem.* 273, 16305–16310
54 De Vries-van Leeuwen, I.J. *et al.* (2013) Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface. *Proc. Natl. Acad. Sci. U. S. A.* 110, 8894–8899
55 De Vries-van Leeuwen, I.J. *et al.* (2010) Fusicoccin-A selectively induces apoptosis in tumor cells after interferon-alpha priming. *Cancer Lett.* 293, 198–206
56 Pei, L. *et al.* (2003) Oncogenic potential of TASK3 (Kcnk9) depends on K^+ channel function. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7803–7807
57 Anders, C. *et al.* (2013) A semisynthetic fusicoccane stabilizes a protein–protein interaction and enhances the expression of K^+ channels at the cell surface. *Chem. Biol.* 20, 583–593
58 Molzan, M. *et al.* (2013) Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. *ACS Chem. Biol.* 8, 1869–1875
59 Bier, D. *et al.* (2013) Molecular tweezers modulate 14-3-3 protein–protein interactions. *Nat. Chem.* 5, 234–239
60 Zhao, J. *et al.* (2011) Discovery and structural characterization of a small molecule 14-3-3 protein–protein interaction inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* 108, 16212–16216
61 Takemoto, Y. *et al.* (2005) Chemistry and biology of moverastins, inhibitors of cancer cell migration, produced by *Aspergillus*. *Chem. Biol.* 12, 1337–1347
62 Sawada, M. *et al.* (2011) Synthesis and anti-migrative evaluation of moverastin derivatives. *Bioorg. Med. Chem. Lett.* 21, 1385–1389
63 Kobayashi, H. *et al.* (2011) Involvement of 14-3-3 proteins in the second epidermal growth factor-induced wave of Rac1 activation in the process of cell migration. *J. Biol. Chem.* 286, 39259–39268
64 Huhtaniemi, I. and Forti, G. (2011) Male late-onset hypogonadism: pathogenesis, diagnosis and treatment. *Nat. Rev. Urol.* 8, 335–344
65 Ikeda, M. *et al.* (2008) Identification of YWHAE, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia. *Hum. Mol. Genet.* 17, 3212–3222
66 Cheah, P.S. *et al.* (2012) Neurodevelopmental and neuropsychiatric behaviour defects arise from 14-3-3zeta deficiency. *Mol. Psychiatry* 17, 451–466
67 Ramshaw, H. *et al.* (2013) Locomotor hyperactivity in 14-3-3zeta KO mice is associated with dopamine transporter dysfunction. *Transl. Psychiatry* 3, e327
68 Toyo-Oka, K. *et al.* (2003) 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller-Dieker syndrome. *Nat. Genet.* 34, 274–285