EXPERT OPINION

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Targeting the liver kinase B1/AMP-activated protein kinase pathway as a therapeutic strategy for hematological malignancies

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Introduction: Despite considerable advances, several hematological malignancies remain incurable with standard treatments. Therefore, there is a need for novel targeted and less toxic therapies, particularly for patients who develop resistance to traditional chemotherapeutic drugs. The liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signaling pathway has recently emerged as a tumor suppressor axis. A critical point is that the LKB1/AMPK network remains functional in a wide range of cancers and could be stimulated by drugs, such as N,N-dimethylimidodicarbonimidic diamide (metformin) or 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR).

Areas covered: The literature data show that drugs activating LKB1/AMPK signaling induced cell cycle arrest, caspase-dependent apoptosis or autophagy in hematopoietic tumors. Moreover, metformin effectively inhibited mammalian target of rapamycin complex 1 (mTORC1)-controlled oncogenetic protein translation, which does not occur with allosteric mTORC1 inhibitors, such as rapamycin and its derivatives. Metformin was also capable of targeting leukemic stem cells, the most relevant target for leukemia eradication.

Expert opinion: Data emerging from preclinical settings suggest that the LKB1/AMPK pathway is critically involved in regulating proliferation and survival of malignant hematopoietic cells. Thus, it is proposed that drugs activating the LKB1/AMPK axis may offer a novel and less toxic treatment option for some types of hematological malignancies.

Keywords: AMPK, leukemias, LKB1, metformin, translation

Expert Opin. Ther. Targets [Early Online]

1. Introduction

The deregulation of signaling and metabolic pathways strongly supports proliferation and survival of cancer cells [1]. In particular, the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK)/mammalian target of rapamycin complex 1 (mTORC1) axis plays a key role in linking metabolism and energy status to signal transduction cascades involved in cell growth, proliferation, survival, and autophagy [1]. The discovery that LKB1 was the major upstream kinase involved in AMPK activation connected for the first time cancer biology with AMPK activity [2]. LKB1, which is encoded by the serine/threonine protein kinase 11 (*STK11*) gene, was originally identified as the tumor suppressor responsible for the Peutz–Jeghers syndrome. Peutz–Jeghers patients display germ-line mutations of *STK11* [3] and suffer from the frequent formation of benign gastrointestinal hamartomatous polyps; however, they also have an increased risk of malignant cancers arising in other organs, including breast, uterus, and lung [4]. Nevertheless, as it could be

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Article highlights.

- The tumor suppressor LKB1/AMPK axis remains functional in a wide range of cancers and could be stimulated by drugs, such as metformin or AICAR.
- LKB1/AMPK signaling negatively affects mTORC1 which is overactivated in many cancer types. Thus, upregulation of LKB1/AMPK activity results in decreased oncogenetic protein translation, cell cycle arrest, apoptosis, and autophagy.
- Drugs activating LKB1/AMPK signaling induced cell cycle arrest, caspase-dependent apoptosis or autophagy in a wide range on hematological cancers (acute and chronic leukemia, multiple myeloma, NHLs).
- Remarkably metformin was cytotoxic to putative leukemic stem cells, the true target for leukemia eradication.
- Recent findings have highlighted LKB1, but not AMPK, as a critical factor in hematopoietic stem cell quiescence.

This box summarizes key points contained in the article.

expected for a tumor suppressor gene, inactivating *STK11* mutations have been detected in sporadic cancers, including ~ 30% of non–small-cell lung carcinomas [5], 20% of cervical cancers [6], and 5% of pancreatic carcinomas and melanomas [4]. LKB1 is a 50-kDa serine/threonine protein kinase, which is ubiquitously expressed in fetal and adult tissues [3]. LKB1 is retained in the nucleus in an inactive state; however, upon phosphorylation and translocation to the cytoplasm, LKB1 is activated following the formation of a heterotrimeric complex with STE20-related protein α (STRADα) and scaffolding mouse 25 protein (MO25) (**Figure 1**) [7-9]. Upon activation, LKB1 phosphorylates and activates AMPK and its related 12 serine/threonine protein kinases [1]. Several lines of evidence suggest that the LKB1/AMPK signal transduction network is functional in a wide variety of neoplasias, including hematopoietic malignant disorders, and that pharmacological activators of this pathway may represent a novel type of targeted therapy [10-15].

In this paper, we will mainly focus on the modulation of LKB1/AMPK signaling in hematological cancers and its implications for clinical trials employing selective activators of this signal transduction axis. However, we shall also provide a general overview of the LKB1/AMPK signaling pathway and of its relationship with mTORC1.

2. The LKB1/AMPK signaling pathway

The serine/threonine protein kinase AMPK is a key cellular energy sensor which is activated by phosphorylation at Thr 172 of its catalytic subunit in response to changes in cellular ATP levels [16]. AMPK is a heterotrimer, composed of a catalytic α subunit and two regulatory (β and γ) subunits (**Figure 1**). In mammalian cells, each subunit has multiple isoforms that are differentially expressed in different tissues. For instance, there are two isoforms of the catalytic α subunit (α1 and α2), while β and γ subunits have two (β1 and β2) and three (γ1, γ2, and γ3) isoforms, respectively [17]. The β subunits provide a structural link between the other subunits and also contain a specialized sequence which binds to glycogen, whereas the γ subunits display the binding sites that enable AMP to activate the complex. The existence of multiple subunit isoforms results in the generation of 12 possible heterotrimeric combinations of AMPK [1].

An increase in cellular AMP/ATP ratio can be the consequence of energy starvation or cellular stresses that decrease ATP production (hypoxia, metabolic poisons, etc.). Indeed, especially AMP and ADP cell concentrations are much lower than those of ATP [18,19]. Therefore, even a small decrease in ATP will result in a relatively large increase in ADP and AMP. Under these conditions, AMP binding causes an allosteric change in the conformation of the AMPK γ subunits, thus exposing the Thr 172 residue on the α subunits for phosphorylation by LKB1. This phosphorylation increases AMPK activity [20]. However, recent findings have highlighted that ADP also promotes AMPKα subunit phosphorylation at Thr 172 [21].

The activation loop of the AMPKα subunits can be phosphorylated by other kinases, including calcium/calmodulin-dependent protein kinase β (CaMKK β) [22], and transforming growth factor β-activated kinase 1 (TAK1) [20,23,24]. Phosphorylation of AMPKα by CaMKK β plays an important role in Ca^{2+}-dependent signaling in T-lymphocytes [25], while TAK1 is a protein kinase acting downstream of cytokine receptors. However, the physiological role of TAK1-regulated AMPKα phosphorylation is at present unclear [1].

Activated AMPK phosphorylates many substrates that switch on alternative catabolic pathways for generating more ATP. Moreover, AMPK phosphorylates some substrates that turn off anabolic biosynthetic pathways for preventing additional ATP consumption. Therefore, activation of the LKB1/AMPK pathway in response to acute metabolic stresses is of paramount importance for the survival of cells as well as whole organisms during energy crises [16]. For example, under hypoxic conditions, AMPK phosphorylates and activates two of the isoforms of 6-phosphofructo-2-kinase (PFK2), the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis [26].

AMPK phosphorylates acetyl CoA carboxylase 1 and 2 (ACC1 and ACC2) [27], two key enzymes for fatty acid synthesis and oxidation [28]. Upon phosphorylation by AMPK, ACC1/2 activity is inhibited. ACC1 phosphorylation leads to suppression of the production of malonyl-CoA, a key signaling molecule in many metabolic pathways [29]. AAC2 phosphorylation relieves inhibition of fatty acid uptake into mitochondria thus enhancing fatty acid oxidation in skeletal muscle. Therefore, active AMPK allows muscle cells to utilize lipids as an alternative source of energy when the cells are not able to obtain energy from carbohydrates [1]. AMPK phosphorylates and inhibits 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of the

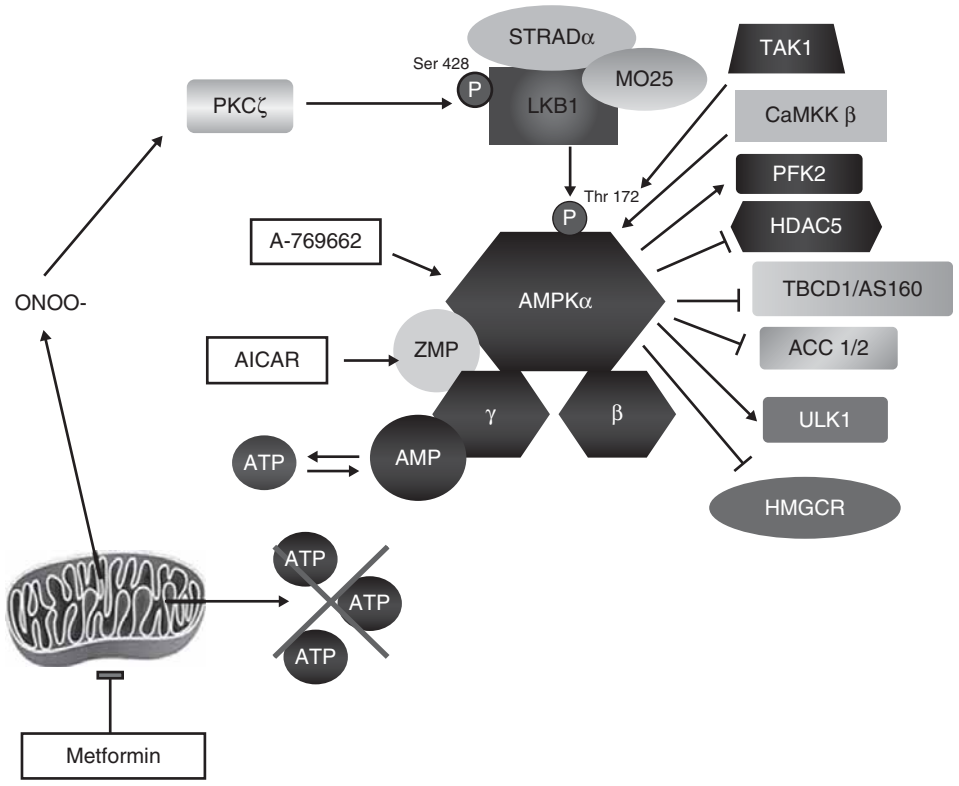

Figure 1. The LKB1/AMPK axis and its activators. Once phosphorylated at Ser 428 by PKCζ, LKB1 migrates from the nucleus to the cytoplasm where it is activated through interactions with STRADα and MO25. Activated LKB1 then phosphorylates AMPKα subunit at Thr 172. However, Thr 172 can be phosphorylated by other kinases, including CaMKK β and TAK1. Activated AMPK targets several proteins that play critical roles in carbohydrate (PFK2, HDAC5, TBCD1/AS160) and lipid (ACC1/2, HMGCR) metabolism, as well as in autophagy (ULK1). Metformin activates AMPK by two LKB1-dependent mechanisms. First, by inhibiting complex I of the mitochondrial respiratory chain, metformin generates reactive nitrogen species ($ONOO^-$) that in turn activate PKCζ. Second, complex I inhibition by metformin decreases mitochondrial ATP production and increases AMP levels, that allosterically activate AMPK. AICAR is phosphorylated by adenosine kinase inside the cells and the resulting product, ZMP, acts as an AMP mimetic which is thought to directly bind the AMP-binding pockets of the AMPKγ subunits. A-769662 is the only known small molecule that directly binds AMPK, inducing its activity; however, the A-769662/AMPK interaction domain is currently unknown. Arrows indicate activating events, whereas perpendicular lines highlight inhibitory events.

ACC 1/2: Acetyl CoA carboxylase 1/2; AICAR: 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMPK: AMP-activated kinase; AS160: Akt substrate of 160-kDa; CaMKK β: Calcium/calmodulin dependent protein kinase β; HDAC5: Histone deacetylase 5; HMGCR: 3-hydroxy 3-methylglutaryl coenzyme A reductase; LKB1: Liver kinase B1; MO25 protein: Mouse 25 protein; $ONOO^-$: Reactive nitrogen species; PFK2: 6-phosphofructo-2-kinase; PKCζ: Protein kinase Cζ; STRADα: STE20-related protein α; TAK1: Transforming growth factor β-activated kinase 1; TBCD1: Tre-2/USP6, BUB2, cdc16 domain family member 1; ULK1: Unc-51- like kinase 1; ZMP: 5-amino-4-imidazolecarboxamide ribotide.

mevalonate pathway, the metabolic pathway that produces cholesterol [30]. AMPK targets cyclic AMP response element binding protein (CREB)-regulated transcriptional coactivator-2 (or CRTC2) [31], and, by doing so, it inhibits expression of genes coding for proteins involved in liver gluconeogenesis, including glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [31,32]. Other downstream targets of AMPK include Rab-GAP (GTPase-activating protein) proteins TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1) and AS160 (Akt substrate of 160-kDa) [33,34], several members of the histone deacetylase (HDAC) family [35] (including HDAC5 [36]), and peroxisomal proliferator-activator receptor (PPAR) γ coactivator-1α (PGC1α) [32]. TBCD1, AS160, and HDAC5 impact directly or indirectly on glucose transporter 4 (GLUT4) and glucose uptake. Indeed, following phosphorylation by AMPK, TBC1D1 and AS160 are inhibited, leading to Rab activation and increased plasma membrane localization of GLUT4 [37,38]. The functional consequence of the AMPK-mediated phosphorylation of HDAC5 is its association with 14-3-3 proteins, nuclear export, and an attenuated interaction of HDAC5 with myocyte enhancer factor 2A. This, in turn, reduces the binding of HDAC5 at the GLUT4 promoter and increases GLUT4 gene expression through an enhanced activity of myocyte enhancer factor 2A [36].

AMPK-dependent phosphorylation of the transcriptional coactivator PGC1α, the master regulator of mitochondrial

biogenesis, is crucial for the coordinated expression of the nuclear-encoded mitochondrial genes [39]. Moreover, AMPK also appears to regulate PGC1α by deacetylation [40].

Protein synthesis is an energetically costing process that requires copious amounts of ATP and GTP, as well as the production of an abundant number of ribosomes. Thus, it is not surprising that AMPK inhibits translation elongation by regulating eukaryotic elongation factor 2 (eEF2) [41], although eEF2 is not a direct substrate of AMPK. Accordingly, AMPK activation resulted in protein synthesis inhibition [14]. However, the effects of AMPK activation on protein synthesis are mostly due to inhibition of mTORC1 activity (see below).

Conversely, autophagy is a process by which cell components and organelles are degraded and recycled to maintain essential activity and viability in response to nutrient limitations. AMPK promotes autophagy by directly activating the unc-51-like kinase 1 (ULK1) through phosphorylation at Ser 317 and Ser 777 [42]. ULK1 is the mammalian homolog of yeast autophagy-specific gene 1 (ATG1) [43]. Some of the AMPK targets are summarized in **Figure 1**.

3. mTORC1

The serine/threonine kinase mTOR is the catalytic subunit of two multiprotein complexes, referred to as mTORC1 and mTORC2 that are defined by their protein partners and substrate specificity [44]. In addition to mTOR, mTORC1 contains the regulatory associated protein of mTOR (Raptor, a scaffolding protein which recruits mTORC1 substrates and regulates mTORC1 assembly), mammalian Lethal-with-Sec-Thirteen 8 (mLST8, also referred to as GTPase β-subunit like, or GβL), PRAS40 (prolin-rich Akt substrate of 40-kDa), and DEP (disheveled, Egl-10 and Pleckstrin)-domain-containing mTOR interacting protein (Deptor) (**Figure 2**). mTORC1 is sensitive to rapamycin and its derivatives (rapalogs) that act as powerful and selective allosteric mTORC1 inhibitors [45]. Two of the most well-characterized mTORC1 substrates are 70-kDa ribosomal protein S6 kinase 1/2 (p70S6K 1/2) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (**Figure 2**). mTORC1 phosphorylates p70S6K 1/2 at Thr 389 [46]. Thr 389 phosphorylation recruits phosphoinositide-dependent kinase-1 (PDK1) and enhances PDK1-dependent Thr 229 phosphorylation in the activation loop of p70S6K 1/2, which is essential for its activation [47]. p70S6K 1/2 phosphorylates several substrates, including S6 ribosomal protein (S6RP), eukaryotic translation initiation factor 4B (eIF4B), programmed cell death 4 (PDCD4), p70S6K 1/2 Aly/REF-like target (SKAR), and eukaryotic elongation factor 2 kinase (eEF2K) (**Figure 2**). All of these substrates are involved in protein translation [48-52]. It is important to underline here that p70S6K 1/2, when activated by mTORC1, directly phosphorylates the insulin receptor substrate-1 and -2 (IRS1 and IRS2), which promotes their degradation and reduces the ability of growth factors to signal downstream of receptor tyrosine kinases, such as insulin receptor (IR) or insulin-like growth factor-1 receptor (IGF-1R) [44,45]. IRS1 and IRS2 are normally required to activate phosphatidylinositol 3-kinase (PI3K)/Akt after stimulation of the IR/IGF-1R tyrosine kinase activity. Therefore, inhibition of mTORC1 signaling could block this negative feedback loop and activate Akt through PI3K [44,45].

4E-BP1 is a member of the 4E-BP family which also includes 4E-BP2 and 4E-BP3. 4E-BP proteins are repressors of translation initiation [53]. 4E-BP proteins display an eukaryotic translation initiation factor 4E (eIF4E) binding domain, which is shared by eIF4G, an essential scaffolding protein. eIF4E, eIF4G, and the ATPase/RNA helicase, eIF4A, form the eIF4F complex [54]. Hypophosphorylated 4E-BP1 strongly interacts with eIF4E, thereby interfering with the binding between eIF4E and eIF4G. Following mTORC1-dependent phosphorylation, 4E-BP1 dissociates from eIF4E, thereby relieving the inhibitory effect on eIF4E-dependent translation initiation [55]. It should be emphasized that 4E-BP1 phosphorylation levels are critical for the translation of mRNAs with long or complex structural elements in their 5'UTRs (untranslated regions). This kind of mRNAs mainly encode prosurvival and anti-apoptotic oncogenetic proteins, including cyclin D1, c-Myc, B-cell leukemia-2 (Bcl-2), vascular endothelial growth factor, and matrix metalloproteinase-9. In contrast, housekeeping protein mRNA translation is less affected by the 4E-BP1 phosphorylative status [56].

mTORC1 prevents ULK1 activation by phosphorylation at Ser 757 (**Figure 2**). This phosphorylative event disrupts the interactions between ULK1 and AMPK, thus repressing autophagy [57,58].

4. LKB1/AMPK signaling and mTORC1 activity regulation

mTORC1 activity is mainly regulated through the PI3K/Akt pathway. Akt phosphorylates 200-kDa tuberous sclerosis 2 (TSC2 or hamartin). TSC2 is a GAP protein which associates with 130-kDa tuberous sclerosis 1 (TSC1 or tuberin) to inactivate the small G protein Ras homolog enriched in brain (Rheb) (**Figure 2**). Once phosphorylated by Akt at Thr 1462 [59], TSC2 binds 14-3-3 proteins [60]. Upon binding to 14-3-3 proteins, TSC2 is degraded by the proteasome, and Rheb accumulates in a GTP-bound state. The mechanism by which Rheb-GTP activates mTORC1 has not been fully elucidated yet, although Rheb requires farnesylation for activating mTORC1 [60]. In contrast, AMPK phosphorylates TSC2 at Thr 1227 and Ser 1345 and activates the TSC1/TSC2 complex (**Figure 2**). Therefore, these phosphorylative events downmodulate mTORC1 signaling [61]. Consistently, mTORC1 activity inhibition by 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), an AMPK activator, or by glucose deprivation, was largely compromised in TSC1- or TSC2-deficient cells [62,63]. Moreover, AICAR failed to inhibit mTORC1 activity in AMPKα1/α2 double-knockout mouse embryonic fibroblasts (MEFs) [64]. Furthermore, others have found that AMPK phosphorylated

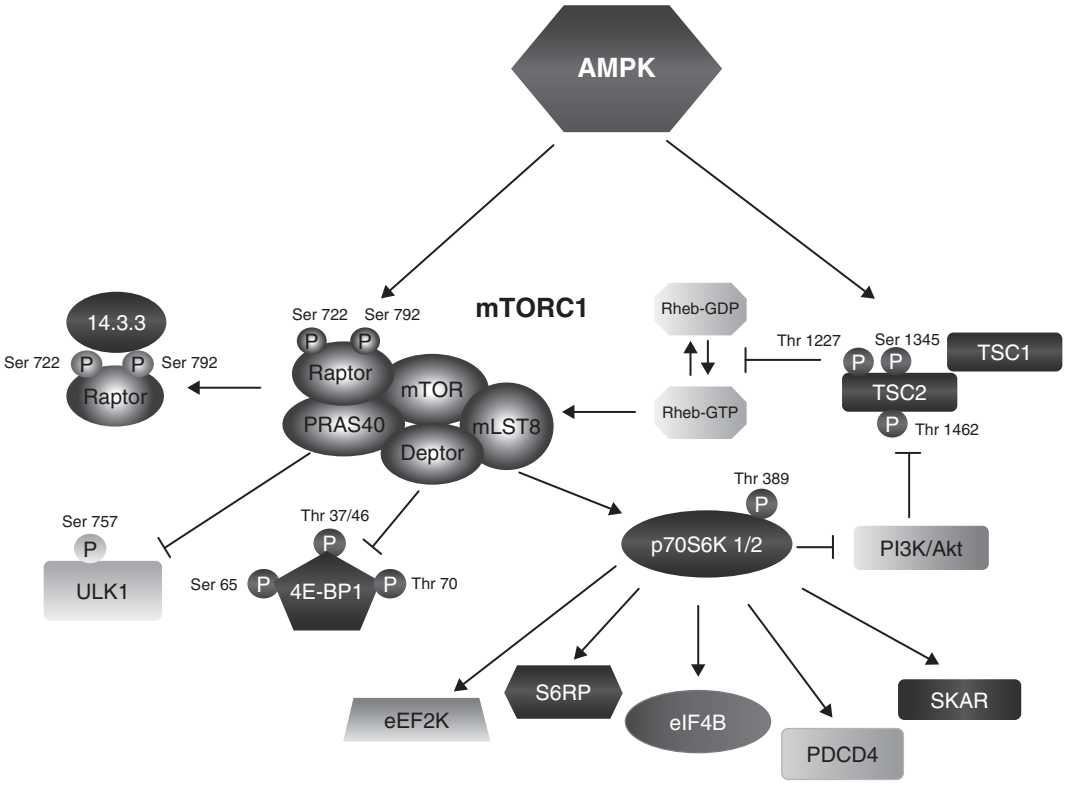

Figure 2. AMPK-mediated control of mTORC1 activity. AMPK phosphorylates TSC2 at Thr 1227 and Ser 1345 and activates the TSC1/TSC2 complex. Therefore, these phosphorylative events repress mTORC1 activity, as they cause Rheb to accumulate in a GDP-bound state. In contrast, once phosphorylated by Akt at Thr 1462, TSC2 binds 14-3-3 proteins. Upon binding to 14-3-3 proteins, TSC2 is degraded by the proteasome, and Rheb accumulates in a GTP-bound state. This results in mTORC1 activation. Moreover, AMPK phosphorylates Raptor at Ser 722 and Ser 792 and these phosphorylative events induce 14-3-3 protein binding to Raptor and mTORC1 disassembly/inhibition. mTORC1 targets 4E-BP1 (a translational repressor) and p70S6K1/2, which increases translation by phsophorylating eEF2K, S6RP, eIF4B, PDCD4, and SKAR. Active (phosphorylated) p70S6K1/2 represses PI3K/Akt signaling through a mechanism which involves IGF-1/IGF-1R/IRS 1/2. mTORC1 also phosphorylates and inhibits ULK1. Arrows indicate activating events, whereas perpendicular lines highlight inhibitory events.

AMPK: AMP-activated kinase; Deptor: Disheveled, Egl-10 and Pleckstrin–domain-containing mTOR interacting protein; 4E-BP1: Eukaryotic initiation factor 4E-binding protein 1; eEF2K: eukaryotic elongation factor 2 kinase; eIF4B: Eukaryotic translation initiation factor 4B; mLST8: Mammalian Lethal-with-Sec-Thirteen 8; mTOR: Mammalian target of rapamycin; mTORC1: mTOR complex 1; p70S6K ½: 70-kDa ribosomal protein S6 kinase 1/2; PDCD4: Programmed cell death 4; PI3K: Phosphatidylinositol 3-kinase; PRAS40: Prolin-rich Akt substrate of 40-kDa; Raptor: Regulatory associated protein of mTOR; Rheb: Ras homolog enriched in brain; S6RP: S6 ribosomal protein; SKAR: p70S6K 1/2 Aly/REF-like target; TSC1: Tuberous sclerosis 1; TSC2: Tuberous sclerosis 2; ULK1: Unc-51-like kinase 1.

Raptor at Ser 722 and Ser 792 and that these phosphorylative events induced 14-3-3 protein binding to Raptor and mTORC1 disassembly (**Figure 2**). Accordingly, cells expressing a phospho-defective Raptor mutant with alanine substitutions at both Ser 722 and Ser 792 were resistant to AICAR-induced mTORC1 inhibition [65]. These observations indicated that AMPK-induced Raptor phosphorylation decreased the mTORC1 activity. However, such a regulatory mechanism could not be detected in all cell types [63].

LKB1-null MEFs, as well as other mouse and human *LKB1*-deficient cells display mTORC1 hyperactivation [66-69]. Moreover, mTORC1 hyperactivation has been detected in intestinal polyps of *LKB1*$^{+/-}$ mouse and Peutz–Jeghers syndrome patients, indicating a key role played by LKB1/AMPK signaling in the control of mTORC1 activity [67,70].

5. Therapeutic modulators of LKB1/AMPK activity

Major efforts have been made to develop pharmacological compounds capable of activating the LKB1/AMPK axis. The biguanide molecule metformin (N,N-dimethylimidodicarbonimidic diamide) is a well-known oral drug that is widely used for treating type 2 diabetes [71]. Metformin activates AMPK by at least two LKB1-dependent mechanisms. First, by inhibiting complex I of the mitochondrial respiratory chain, metformin generates reactive nitrogen species that in turn activates protein kinase C (PKC)ζ. PKCζ then phosphorylates LKB1 at Ser 428 which is required for LKB1 nucleo-cytoplasmic translocation and subsequent AMPK activation [72]. Second, complex I inhibition by metformin

increases intracellular AMP levels that allosterically activate AMPK (**Figure 1**) [73,74].

Nevertheless, recent findings have highlighted that metformin-induced mTORC1 inhibition could be independent of AMPK [64]. This work demonstrated that metformin inhibition of mTORC1 activity depended on Ras-related GTPases (Rag), but the exact molecular mechanisms by which metformin modulated the Rag-mTORC1 pathway remain unclear. In any case, in the absence of amino acids, mTORC1 is diffusely localized throughout the cytoplasm, whereas in the presence of amino acids, Rag proteins bind Raptor and target mTORC1 to a Rab7-positive late endosomal compartment containing Rheb-GTP, which then activates mTORC1 [75,76].

Phenformin (2-(N-phenethylcarbamimidoyl)guanidine) is another oral anti-diabetic drug of the biguanide class. Phenformin is more powerful than metformin in activating AMPK (1). However, the use of phenformin as an antidiabetic drug was discontinued in the 1970s, as phenformin caused 64 cases of lactic acidosis per 100,000 patients/year, compared to 3 cases per 100,000 patients year caused by metformin [77].

The thiazolidinedione, rosiglitazone, is an antidiabetic drug which activates AMPK. Rosiglitazone works as an insulin sensitizer, by binding to PPARγ in adipocytes [78]. In analogy with metformin, rosiglitazone induced an increase in the AMP:ATP ratio and AMPKα subunit phosphorylation [74,79,80]. Pioglitazone is another thiazolidinedione which is capable of activating AMPK [81]. However, rosiglitazone, but not pioglitazone, use in diabetic patients has been associated with increased cardiovascular risk [82].

AICAR is a cell-permeable compound that is phosphorylated by adenosine kinase inside the cells and the resulting product, 5-amino-4-imidazolecarboxamide ribotide or ZMP, acts as an AMP mimetic which is thought to directly bind the AMP-binding pockets of the AMPKγ subunits (**Figure 1**) [83-85].

The A-769662 compound is the only known small molecule that directly binds AMPK, inducing its activity, although the A-769662/AMPK interaction domain is currently unknown [86].

AMPK can also be activated by a wide range of 'xenobiotics' that include resveratrol (from red wine), epigallocatechin gallate (from green tea), and some plant products (galegine, berberine, hispidulin) [1]. Some of these compounds inhibit mitochondrial ATP synthesis, by targeting complex I of the respiratory chain (e.g., berberine, [87]) or the ATP synthase, complex V (e.g., resveratrol [88]); hence, their mechanism of action on AMPK is similar to metformin [74].

6. AMPK activation, cancer prevention, and therapy

Epidemiological studies performed over the last 7 years have documented that metformin has antineoplastic properties. It was found that diabetics patients that had been treated with metformin displayed a significantly lower incidence of cancers than those on other treatments (e.g., secretagogue drugs) [89]. Since then, these results have been confirmed in other studies [90-92], including a meta-analysis investigation [93].

How could metformin prevent tumor development *in vivo*? It has been hypothesized that metformin, by inhibiting liver gluconeogenesis (at least partly through the activation of AMPK), lowers plasma glucose, insulin, and possibly, free IGF-1, a powerful growth factor for many types of neoplastic cells [94]. These events would indirectly oppose tumorigenesis. Moreover, metformin could activate AMPK directly in developing tumors, thus exerting a cytostatic effect [1]. Type 2 diabetic patients are often overweight or obese, two conditions that favor the incidence of cancers [95]. Moreover, hyperinsulinemia and type 2 diabetes are associated with an increased risk of many cancer types [96-98].These findings could support the first hypothesis regarding the mechanisms of action of metformin in preventing carcinogenesis. However, the second hypothesis is supported by the observation that three different AMPK activators (metformin, phenformin, and A-769662) significantly delayed tumor development in tumor-prone mice that were not diabetic or insulin-resistant [99]. Interestingly, AMPK activity was found to be downregulated, when compared to normal epithelial cells, in 90% of 350 patients with breast cancer [100]. As outlined above, inactivating mutations of the *STK11* gene are quite common in some cancer types; however, another more general mechanism for AMPK inactivation in tumors could be represented by the fact that Akt-dependent Ser 485 phosphorylation on AMPKα1 or Ser 491 phosphorylation on AMPKα2 inhibited subsequent phosphorylation at Thr 172 by LKB1 [101]. Activation of the PI3K/Akt pathway is extremely frequent in a wide variety of cancers [102]; hence, it could be a major cause for AMPK inactivation.

There is a plethora of preclinical studies that have documented the *in vitro* and *in vivo* antitumor growth properties of AMPK activating drugs, such as metformin and AICAR, in a wide range of neoplasias, that include breast, colon, prostate, ovarian, and lung carcinoma (reviewed in [103-106]). The remarkable efficiency of metformin to inhibit neoplastic cell growth *in vitro* and tumor proliferation in animals *in vivo*, and its low toxicity, have provided the rational for clinical trials in which cancer patients (especially breast cancer patients) are being treated with metformin alone or in combination with other antineoplastic drugs (see clinicaltrials.gov).

7. Therapeutic targeting of LKB1/AMPK signaling in malignant hematopoietic disorders

Compared to the extensive research performed on LKB1/AMPK modulation in solid tumors, limited work has been done so far in hematological cancers.

7.1 Chronic lymphocytic leukemia

In 2003, Campas *et al.* [10], for the first time documented that AICAR induced a caspase-dependent, but p53-independent, apoptotic cell death in chronic lymphocytic leukemia (CLL) primary cells. CLL affects B-lymphocytes and is the most common form of chronic leukemia [107]. The IC_{50} of AICAR in CLL lymphocytes was about 380 μM. AICAR induced the phosphorylation of AMPKα in CLL cells. Moreover, nitrobenzylthioinosine (a nucleoside transport inhibitor), 5-iodotubercidin (an inhibitor of adenosine kinase), and adenosine completely inhibited AICAR-induced apoptosis and AMPK phosphorylation. These findings demonstrated that incorporation of AICAR into the cells and its subsequent phosphorylation to ZMP by adenosine kinase were necessary events to induce apoptosis [10]. Although T-lymphocytes from CLL patients were only slightly affected by AICAR at doses up to 4 mM, healthy B-lymphocytes were as sensitive as B-CLL cells to AICAR-induced apoptosis. This finding suggested that at least cellular immunity could be preserved if CLL patients were to be treated with AICAR. However, recent work from the same group has highlighted that AICAR induced apoptosis of CLL B-lymphocytes also through the upregulation of the three Bcl-2 homology domain 3 (BH3)-only proteins: Bcl-2-like protein 11 (BIM), Noxa, and p53 upregulated modulator of apoptosis (PUMA). These results demonstrated the existence of an AMPK-independent pathway of apoptosis induction elicited by AICAR in CLL B-lymphocytes [108]. It is worth emphasizing here that BIM, Noxa, and Puma are powerful pro-apoptotic members of the Bcl-2 family of proteins [109]. Interestingly, a Phase I clinical trial of AICAR in CLL patients is now undergoing (NCT00559624).

7.2 Chronic myelogenous leukemia

AICAR was cytotoxic to K562, LAMA-84, and JURL-MK1 chronic myelogenous leukemia (CML) cell lines and was also effective in killing imatinib-resistant K562 cells and mouse Ba/F3 cells carrying the T315I Bcr-Abl mutation [110]. It should be reminded that T315I Bcr-Abl mutants are resistant to tyrosine kinase inhibitors (imatinib, nilotinib), that represent the treatment of choice for CML patients [111]. AICAR induced an AMPK-independent autophagy process in CML cell lines, but not apoptosis. It was also assumed that AICAR-mediated autophagic cell death was dependent upon PKC activation. However, no identification of PKC isoform(s) involved in the process was possible. Moreover, AICAR was effective in a xenograft model of K562 cells in nude mice [110]. Others have reported that both AICAR and metformin were cytotoxic *in vitro* to CML cells; however, they did not fully address the importance of AMPK activation for the cytotoxic effects [112]. Indeed, the authors did not document AMPKα subunit phosphorylation in response to either AICAR or metformin, but they just showed increased phosphorylation of ACC1, as well as dephosphorylation of p70S6K, S6RP, and 4E-BP1. Furthermore, when the effects of AMPK activators on $CD34^+$ leukemic stem cells (LSCs) were examined, a potent, dose-dependent, suppression of leukemic colony formation from different CML patients was observed [112].

The xenobiotic resveratrol stimulated AMPK and induced autophagy in CML cells. The effects of resveratrol were at least partly abrogated by knocking down AMPKα1/α2, indicating that AMPK mediated the antileukemic activity of resveratrol in CML. Moreover, resveratrol caused autophagic cell death in $CD34^+$ LSCs from CML patients [13,113].

Overall, the above-outlined papers suggested that AMPK agonists may therefore have a role in CML treatment, particularly in case of resistance to tyrosine kinase inhibitor therapy. However, some of the reported antineoplastic effects seem to be AMPK-independent.

7.3 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) can arise from progenitors of either B-lymphocytes (B-ALL) or T-lymphocytes (T-ALL) [114]. As to B-ALL, Sengupta *et al.* [115] demonstrated that AICAR displayed antiproliferative and pro-apoptotic effects *in vitro* against a panel of B-ALL cell lines. Since these effects were abolished by treatment with 5'-iodotubericidin, it was concluded that AICAR cytotoxicity was mediated through AMPK activation. Combined treatment with AICAR and the mTORC1 inhibitor, rapamycin, resulted in additive antiproliferative activity in B-ALL cells, suggesting that AICAR did not block all of the mTORC1 outputs. Moreover, these authors reported that AICAR treatment resulted in increased Akt phosphorylation. Compensatory upregulation of PI3K/Akt signaling is generally observed as a result of mTORC1 inhibition, because under normal conditions activated mTORC1 restrains further activation of PI3K/Akt [116]. It is well established that this negative feedback loop is dependent on p70S6K/IGF-1/IGF-1R/IRS1 or IRS2 signaling (**Figure 2**) [117]. Accordingly, Akt phosphorylation at Thr 308 was not detected if, in addition to AICAR, B-ALL cells had been treated with the tyrosine kinase inhibitor HNMPA(AM)3, an IGF-1R inhibitor [118]. Consistently, combined therapy simultaneously targeting IGF-1R and AMPK resulted in synergistic growth inhibition and cell death. The combination of AICAR plus methotrexate (MTX, a drug widely employed for treating B-ALL patients [119]), induced B-ALL cell death predominantly through a sustained endoplasmic reticulum (ER) stress response [120]. However, the MTX plus AICAR combination, dramatically increased p-Akt levels in B-ALL cells. Under these conditions, the concomitant inhibition of Akt (a well-established AMPK signaling antagonist, see [101]) led to further upregulation of AMPK activity and alleviated MTX plus AICAR-induced ER stress and apoptosis. These findings suggested that the effects of AMPK activation on cell death or survival could be significantly different depending on signaling cross-talks with related oncogenetic pathways and provided a new insight into the reported paradoxical prosurvival versus proapoptotic effects of AMPK activation [121]. Moreover, it should be considered that MTX could enhance the

production of ZMP from AICAR, resulting in additional AMPK activation [122]. A clinical trial is underway in which metformin was combined with vincristine, dexamethasone, doxorubicin and PEG–asparaginase for relapsed childhood B-ALL (NCT01324180).

We recently explored the therapeutic potential of metformin against both T-ALL cell lines and primary samples from T-ALL patients, displaying mTORC1 activation. Metformin affected T-ALL cell viability by inducing autophagy and apoptosis. However, it was much less cytotoxic against proliferating $CD4^+$ T-lymphocytes from healthy donors. Western blot analysis demonstrated that metformin increased phosphorylation of PKCζ, AMPKα, and Raptor, while it dephosphorylated mTORC1 downstream targets, p70S6K and 4E-BP1. However, metformin increased Akt phosphorylation in some, but not all, T-ALL cell lines. Increased Akt phosphorylation was dependent on IGF-1/IGF-R1/IRS2/PI3K signaling. At variance with rapamycin, we found a marked inhibition of mRNA translation in T-ALL cells treated with metformin, which impacted on the levels of oncogenetic proteins, including c-Myc and Bcl-X_L. Remarkably, metformin targeted a putative LSC subpopulation ($CD34^+/CD7^-/CD4^-$) in patient samples [15]. PI3K/Akt/mTORC1 activation is very common in T-ALL and portends a poor prognosis [123]. It has been hypothesized that in a mouse model of T-ALL, one of the mechanisms leading to mTORC1 upregulation in T-ALL is miR-19 overexpression. Indeed, miR-19 leads to downregulation of the *Prkka1* gene, which encodes for the AMPKα1 subunit, a potential antagonist of mTORC1 activity [124]. However, our unpublished results have documented that the levels of the AMPKα1 subunit are similar in human T-ALL lymphoblasts when compared to T-lymphocytes from healthy donors.

7.4 Acute myelogenous leukemia

It was recently demonstrated that the LKB1/AMPK axis was consistently functional in acute myelogenous leukemia (AML) cells (both primary patient samples and cell lines) and that AMPK upregulation, using metformin, A-769662, or a dominant activated form of AMPKγ, fully inhibited mTORC1 downstream signaling, as documented by dephosphorylation of p70S6K at Thr 389 and 4E-BP1 at Thr 37/46 [14]. As a result, oncogenetic (c-Myc, cyclinD1, and Bcl-xL) protein synthesis was markedly reduced. Moreover, incubation of AML cells with either metformin or A-769662 resulted in caspase-dependent apoptotic cell death, and this effect was in part reverted by TSC2 knockdown, demonstrating the importance of the TSC2/mTORC1 pathway in the negative survival input resulting from AMPK activation. Importantly, metformin was found not to induce significant levels of apoptosis in healthy $CD34^+$ hematopoietic progenitors. Furthermore, it was documented that metformin exerted a significant antileukemic activity *in vivo* in mice transplanted with the OCI-AML3 human AML cell line, strongly suggesting that AMPK agonists may represent a new perspective for AML therapy [14].

7.5 Non-Hodgkin lymphomas (NHL)

Campas *et al.* reported the use of AICAR in primary samples from follicular lymphoma, mantle cell lymphoma (MCL), and splenic marginal zone B-cell lymphoma. AICAR dose-dependently decreased viability of the samples tested [125].

More recently, Drakos *et al.* documented that in MCL cells, p53 stabilization caused AMPK activation and inhibited mTORC1 signaling, thereby inducing cell cycle arrest and apoptosis. They further demonstrated that AICAR-induced AMPK activation led to mTORC1 inhibition, suggesting a critical role for the p53/AMPK/mTORC1 axis in MCL cells survival and emphasizing AICAR as a possible therapy in MCL [12]. It is worth recalling here that activated (i.e., Ser 15 phosphorylated) p53 is capable of inducing AMPK upregulation, which, in turn, results in mTORC1 downregulation. The p53 could control AMPK activity through sestrin 1 and 2, two proteins that accumulate in cells exposed to genotoxic stress [126].

A Phase I clinical trial (NCT00659568) has studied the side effects and best dose of metformin when given together with the mTORC1 inhibitor, CCI-779 (temsirolimus), in patients with advanced stage non-Hodgkin lymphomas (NHL). However, the results of this trial have not been released yet.

7.6 Multiple myeloma (MM)

Baumann *et al.* [11] treated multiple myeloma (MM) cell lines with AICAR and observed increased AMPKα phosphorylation along with S-phase cell cycle arrest and growth inhibition. These effects were reverted by the AMPK inhibitor, compound C. However, AICAR was a weak inductor of apoptosis in most MM cell lines. Importantly, AICAR blocked proliferation of MM cell lines cultured in HS-5 bone marrow stromal cell conditioned medium. It is established that HS-5 cells produce a range of cytokines (e.g., IGF-1, interleukin-6) that are important for MM cell proliferation, as they upregulate several key signal transduction pathways, including PI3K/Akt/mTORC1 [127]. AICAR also decreased viability of primary cells from MM patients. The thiazolidinedione troglitazone, which is known for activating AMPK [128], similarly inhibited MM cell growth, activated AMPK, and decreased p70S6K phosphorylation levels [11]. These findings suggested that upregulation of AMPK could represent a potential new strategy in the therapy of MM.

8. Conclusions

The LKB1/AMPK axis has been extensively studied in metabolic diseases and more recent evidence suggests its involvement in cancer cell biology. Somatic mutations in the *STK11* gene coding for LKB1 are detected in sporadic human cancers, emphasizing LKB1 as a tumor suppressor. Moreover, pharmacological activation of the LKB1/AMPK axis using metformin, AICAR, or A-769662 compound induced a dramatic suppression of cancer cell growth both *in vitro* and in *in vivo* models of xenografted tumors, demonstrating that activation of

LKB1/AMPK signaling could represent a valuable therapeutic strategy for neoplasias. Several clinical studies are ongoing or have been completed, in a perspective to assess the therapeutic impact of metformin in patients with solid cancers. Very recently, the first paper documenting an *in vivo* reduction of cancer cell proliferation in breast cancer patients treated with metformin has been published [129]. However, AMPK agonists have different molecular targets and display off-target effects. For example, in prostate cancer cells, metformin displayed cytotoxicity independently of AMPK [130].

Similar findings have been reported for AICAR-treated Jurkat T-ALL cells [126]. Therefore, the interpretation of the molecular effects of the currently available AMPK activators is problematic. The spectrum of off-target effects could be reduced with A-769662, as A-769662 directly targets AMPK, in contrast to AICAR or metformin, although this assertion remains to be experimentally verified. Moreover, AMPK substrates are multiple, acting on glucose, fatty acids, and protein metabolism, and the exact AMPK-dependent pathways responsible for tumor suppression remain to be fully elucidated. Several studies have focused on the control of oncogenetic protein translation to explain the tumor suppressive effects of AMPK-activating molecules. These observations appear very intriguing, as the very limited therapeutic efficacy of rapamycin/rapalogs in cancer patients *in vivo* has been attributed also to the lack of effects displayed by these drugs on mTORC1-regulated translation [56]. For this reason, dual PI3K/mTOR inhibitors or ATP-competitive mTORC1/mTORC2 inhibitors are being considered as a more effective way of targeting mTORC1-dependent protein translation in cancer cells [131-133]. However, the *in vivo* toxicity of these new agents that target more globally mTORC1 activity is at present unknown, whereas metformin toxicity is very low.

Although mTORC1-dependent protein translation is consistently overactivated in neoplastic cells, AMPK has many other targets that could be involved in cancer cell proliferation and survival. For example, the overexpression of lipogenic enzymes such as the direct AMPK substrates, ACC1/2, is a common feature of cancers and the specific knockdown of ACC1/2 inhibited cell proliferation and induced caspase-dependent apoptosis in prostate and breast cancer cell lines [134,135]. Therefore, an extensive study of the importance of ACC1/2 or other AMPK downstream substrates in hematological malignancies is necessary to fully understand the effects of metformin and other AMPK agonists. Major efforts have still to be done to elucidate the potential of AMPK agonists as a new perspective for therapy in hematopoietic cancer patients. The development of new molecules selectively activating AMPK, following an empirical or a systematic screening, will be a critical step toward that goal.

9. Expert opinion

Over the last decade, there have been remarkable advances in defining the importance of the LKB1/AMPK signaling pathway in the regulation of events that are critical for tumor cell survival and proliferation. This signaling cascade is an appealing target for a variety of hematological tumors, also because of its inhibitory effect on mTORC1 signaling pathway and oncogenetic protein translation. However, it is also becoming evident that this pathway is extremely complex and that there are events that are dependent on LKB1 but independent of AMPK. This is exemplified by recent findings that have highlighted LKB1 as a critical factor in hematopoietic stem cell (HSC) quiescence. HSCs give rise to all the mature blood lineages and require the maintenance of a delicate balance between quiescence, self-renewal, and differentiation [136]. Three reports, published in 2010 in the same issue of *Nature*, have documented the importance of LKB1 to maintain HSC quiescence [137-139]. All three studies observed that, upon LKB1 deletion, there was an immediate loss of HSC quiescence and increased HSC proliferation. However, this was followed by a progressive decrease in HSC number and by pancytopenia. These findings closely resembled those reported for HSC with upregulated mTORC1 signaling due to phosphatase and tensin (PTEN) homolog depletion [140]. However, at variance with the results obtained with PTEN mutant HSCs, rapamycin failed to rescue HSC depletion even if LKB1 mutant HSCs displayed decreased AMPKα phosphorylation and increased S6RP phosphorylation levels that were decreased by rapamycin administration. Moreover, neither metformin nor A-769662 could rescue the phenotypes exhibited by LKB1 mutant HSCs [138,139]. Furthermore, simultaneous deletion of both AMPK catalytic subunits, α1 and α2, did not result in the same phenotype as LKB1 deletion [137]. Taken together these findings documented that LKB1 regulates HSC quiescence through mechanisms that are independent of both APMK and mTORC1. Since eradication of human leukemia entails the ablation of LSCs [141,142], it will be very critical to establish if LSC survival is dependent on LKB1 alone or on LKB1/AMPK. This could indeed allow for a selective targeting of LSCs by means of AMPK activators. In this connection, it is intriguing that our results have indeed documented the efficacy of metformin on putative T-ALL LSCs [15].

The effects of AMPK activators in the reduction of tumorigenesis have been established in several preclinical settings. In particular, the AMPK activators, metformin and AICAR, have been the subject of many recent studies in various tumor models, starting from efforts to establish their inhibitory effects on cell lines and animal models, ultimately leading to clinical trials in cancer patients. AICAR is well tolerated by healthy individuals, when given intravenously [143], achieving plasma concentrations in the same range as those inducing apoptosis in CLL cells [125]. However, AICAR displays a short half-life after intravenous administration and is not active after oral administration. Moreover, its use is typically accompanied by an increase in blood levels of both lactic and uric acid, making it a poor candidate for long-term use in humans [143].

The novel compound, A-769662, identified from a large chemical screen, might represent an attractive, more direct method of targeting AMPK, as it has a significantly lower IC_{50} than either metformin or AICAR and has been documented to induce AMPK activation at concentrations lower than AMP [86]. Therefore, studies using A-769662 and other similar compounds that may emerge in the future are warranted and might provide effective new tools for targeted treatments of hematological malignancies.

Declaration of interest

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