Trends in Pharmacological Sciences

Targeting methionine aminopeptidase 2 in cancer, obesity and autoimmunity

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Abstract:	For over three decades, methionine aminopeptidase 2 (MetAP2) has been a tentative drug target for the treatment of cancer, obesity and autoimmune diseases. Currently, no MetAP2 inhibitors (MetAP2i) have reached the clinic yet, despite considerable investment by major pharmaceutical companies. Here, we summarize the key series of MetAP2i developed to date, and discuss their clinical development, progress and issues. We coalesce the currently disparate knowledge regarding MetAP2i mechanism of action, and discuss discrepancies across varied studies. Finally, we highlight the current knowledge gaps that need to be addressed to enable successful development of MetAP2 inhibitors in clinical settings.

Highlights/Trends

- Spanning decades, pharmacological inhibition of MetAP2 has been pursued to treat diseases including cancer, obesity and inflammatory diseases.
- Irreversible MetAP2 inhibitors have consistently failed in clinic trials due either to lack of efficacy, or side-effects undermining efficacy, some of which may be due to off-target activity.
- Limited information regarding mode of action and a lack of consistency between clinical applications and drug development efforts have hindered the progress of MetAP2 inhibitors.
- Mechanistic insight into MetAP2 inhibitor mode-of-action, particularly a clear understanding of the MetAP2 substrates driving efficacy, will guide the safe application of MetAP2 inhibitors to treat human disease.

Outstanding questions

- MetAP2 has driven striking pharmacological interest despite lack of clear clinical success, and after three decades and over 15 series of chemically distinct compounds, our knowledge on the mechanism of action of MetAP2 inhibitors (MetAP2i) is still surprisingly unclear. How do the different phenotypic effects attributed to MetAP2 inhibition (cell-specific inhibition of cell proliferation, cytotoxicity, immune modulation, weight loss, and defects in embryonic development) interconnect beyond a shared target? Most studies focus on one phenotypic effect without assessing the impact of MetAP2 inhibition more generally, hindering integration of data and posing more questions than answers.
- What are the molecular mechanisms driving each of these phenotypes, and which mechanisms are shared between phenotypes? Wnt/PCP pathway inhibition seems unlikely to be responsible for the clear p53-dependent cell cycle arrest observed in certain cell types, although both seem to play a role in blocking angiogenesis. The molecular mechanisms of MetAP2i-induced weight loss or immune modulation remain to be elucidated, and appear to be tissue-specific.
- Is there a preference for a specific phenotype in each cell/tissue type and how is this driven? Could differential tissue distribution of compounds be responsible for the semi-selective effect some compounds have shown in clinical trials? And can this be exploited to design MetAP2 inhibitors with a preferred phenotype or therapeutic spectrum?
- The substrate scope of MetAP2 remains very poorly defined, and depends on factors beyond the primary sequence. What are the substrates of MetAP2 in cells and specific tissues? To what extent are substrates rescued by MetAP1 activity? And which substrates drive each MetAP2 inhibition phenotype?
- Can better understanding of MetAP2 biology and MetAP2i mode of action provide improved biomarkers for patient selection, identify novel indications, and lead to science-driven drug combinations in oncology?

Title

Targeting methionine aminopeptidase 2 in cancer, obesity and autoimmunity

Authors

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Keywords

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Abstract

For over three decades, methionine aminopeptidase 2 (MetAP2) has been a tentative drug target for the treatment of cancer, obesity and autoimmune diseases. Currently, no MetAP2 inhibitors (MetAP2i) have reached the clinic yet, despite considerable investment by major pharmaceutical companies. Here, we summarize the key series of MetAP2i developed to date, and discuss their clinical development, progress and issues. We coalesce the currently disparate knowledge regarding MetAP2i mechanism of action and discuss discrepancies across varied studies. Finally, we highlight the current knowledge gaps that need to be addressed to enable successful development of MetAP2 inhibitors in clinical settings.

Glossary

Angiogenesis: the physiological process by which new blood vessels are formed from preexisting vessels, which also plays an important role in supplying nutrients and oxygen for cancer progression and metastasis.

Biotinylation (of compounds): biotin is also known as vitamin B_7 and is involved in normal cell homeostasis. However, in biochemical applications it is widely used for its strong interaction with streptavidin, allowing for efficient enrichment of biotinylated compounds and molecules by affinity purification.

Chorioallantoic membrane model: the highly vascularized membrane found in avian eggs, used as a robust experimental platform to study blood vessels and angiogenesis with relation to cancer research and drug development.

Cytostatic: chemical compound or cellular component that inhibits cell growth; specifically, does not imply or require cytotoxicity.

Cytotoxic: chemical compound that negatively affects cell viability, i.e. toxic for cells.

iMet: initiator methionine resulting from the start codon (typically AUG); the first residue in all proteins in eukaryotes, except in certain organelle-produced proteins where the first residue is formylmethionine, as occurs in prokaryotes.

HUVEC: human umbilical vein endothelial cells, primary cells used as a model for most experiments studying the cytostatic effect of MetAP2 inhibitors

LLE: lipophilic ligand efficiency, a parameter used in drug discovery that links potency (IC_{50}) and lipophilicity (LogP), serving as an estimate of compound quality and developability. LLE = pIC_{50} – LogP, such that compounds with high LLE values are considered of high quality.

Parenteral administration: a blanket term referring to all routes of administration which are not oral, typically involving injection into different types of tissues.

Wnt signalling pathways: The set of cellular signalling cascades that are initiated by binding of Wnt proteins to the frizzled family of receptors. The canonical Wnt pathway or Wnt/β-catenin pathway is characterised by a resulting accumulation of intracellular β-catenin and its translocation to the nucleus, where it regulates gene expression of target genes by direct

binding to transcription factors. Non-canonical Wnt pathways are defined as all other signalling cascades initiated by Wnt binding to frizzled receptors which do not lead to accumulation of β-catenin. These are not as well-defined as the canonical Wnt pathways but are generally divided into two categories: Wnt/Ca^{2+} and Wnt/Planar cell polarity (PCP), depending on the mediators and the final cell responses that are activated. Wnt/Ca^{2+} has been described to affect gene expression through the NFAT transcription factor family, while Wnt/PCP leads to cytoskeletal rearrangement and cell polarity adjustments and promotes cell survival [1].

MetAP2, a highly coveted drug target

Methionine aminopeptidase 2 (MetAP2, Figure 1) has been a sought-after drug target for over three decades, with multiple pharmaceutical companies competing in the quest for potent MetAP2 inhibitors (MetAP2i) for the treatment of cancer, and more recently, obesity and autoimmunity [2–4]. Over ten distinct MetAP2 inhibitor series have been described to date, including both covalent and reversible binders [2,5,14,6–13] (Figure 2, see also Box 1). Although none of these inhibitors has yet reached the market, continued promising preclinical and clinical efficacy results for MetAP2i over the years have retained the interest of the drug development community. Recently, Merck KGaA published the first reversible clinical candidate targeting MetAP2 [15] indicating that the field of MetAP2 inhibitors remains vibrant and of high interest.

Despite this extensive drug development effort, scientific understanding of MetAP2i mode-of-action is surprisingly limited. MetAP2i are strikingly well-tolerated in patients with little or no toxicity at therapeutically relevant doses, and have been developed for impressively diverse pharmacological applications, ranging from treatment of cancer [2,14,16,17], to obesity [4,18], as well as modulation of autoimmunity [19,20]. However, there is currently no mechanistic link between these applications beyond MetAP2 as the putative target.

In this review, we summarize the key MetAP2 inhibitor series developed to date and highlight their clinical development, including several important clinical trial failures. Moreover, we bring together the seemingly disparate mechanistic knowledge, currently distributed across the medicinal chemistry literature, and discuss key discrepancies and knowledge gaps that need to be addressed to support the success of MetAP2i in clinical settings.

Three decades of MetAP2 inhibitors

Fumagillin and fumagillin-like irreversible MetAP2 inhibitors for the treatment of cancer

Interest in MetAP2 as a drug target started in the 1990s with the serendipitous discovery that the fungal mycotoxin fumagillin **1** (Figure 2) exerted strong antiproliferative effects at low micromolar potency in endothelial cells and inhibited angiogenesis (see Glossary) [2]. Fumagillin was then shown to suppress tumour-induced angiogenesis and tumour growth in

a dorsal air sac tumour model in mice [2]. However, it also induced severe weight loss [2]. Takeda pioneered the development of MetAP2i by developing the first fumagillin-derivatives, attempting to preserve anti-tumour activity while avoiding weight loss. From the reported derivatives, TNP-470 **2** (*O*-(chloroacetylcarbamoyl)fumagillol, initially reported as AGM-1470) was the most promising, showing an endothelial cell proliferation inhibition EC_{50} of 40 pM and exerting strong anti-tumour effects in a wide range of solid tumours [2,3,21]. Importantly, the cytostatic effect was preserved across a wide range of concentrations and cytotoxicity was only observed for concentrations of 75 μM and higher, suggesting cytotoxicity might be an off-target effect [21]. In 1992, Takeda started clinical trials with TNP-470 for the treatment of solid tumours [16]. Independent preclinical studies showed promise for fumagillin and TNP-470 against cancers including hemangioendothelioma [22], mesothelioma [23], neuroblastoma [24] and cholangiocarcinoma [25], as well as for joint inflammation and arthritis [26].

In 1997 and 1998, two independent studies demonstrated that MetAP2 is the biological target of both TNP-470 and a structurally related natural product, ovalicin **3** [27,28], another fungal product originally studied for its immunosuppressive properties [29,30]. Subsequent studies confirmed anti-angiogenic and immunosuppressive effects of both ovalicin and TNP-470 [28,31]. Covalent modification of MetAP2 inhibited catalytic activity without interfering with eIF2α phosphorylation (Box 1) [28], confirmed by a crystal structure revealing a covalent bond between His231 in human MetAP2 and the carbon of the spirocyclic epoxide ring of fumagillin [32,33]. Moreover, these studies revealed that the exquisite selectivity of fumagillin and its analogues for MetAP2 over MetAP1 is driven by steric clashes and excessive distancing of key binding residues in MetAP1 [32]. MetAP2 engagement by TNP-470 was shown to correlate with proliferation inhibition in cell culture assays using biotinylated fumagillin [34], directly linking phenotype to inhibition.

Clinical development of TNP-470 was abandoned in the early 2000s after strong neurotoxic adverse effects appeared during Phase III [35]. By this stage, other pharmaceutical companies and academic groups had started to develop novel fumagillin derivatives or potent drug-like reversible MetAP2i (see below). Caplostatin, consisting of TNP-470 conjugated to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers that prevented permeation through the blood brain barrier, reduced neurotoxic adverse effects [35,36] but did not solve the poor oral

bioavailability of fumagillin derivatives, which required parenteral administration [36]. Novel orally available formulations were pursued, including Lodamin (by conjugating TNP-470 to a different copolymer) [35] and PPI-2458 **4**, a new fumagillin derivative developed by Praecis Pharmaceuticals (later acquired by GSK) [10]. PPI-2458 went into clinical trials for the treatment of solid cancers and non-Hodgkin's lymphoma (clinicaltrials.gov, ID: NCT00100347), and against joint inflammation [37,38]. More 'drug-like' fumagillin analogues were also reported, such as spiroepoxytriazole **5** [39].

Reversible MetAP2 inhibitors for the treatment of cancer

In 2003, the first reversible MetAPi was reported by Novartis. LAF389 **6**, a synthetic pro-drug analogue of naturally occurring bengamides, was shown to be a potent dual MetAP1 and MetAP2 inhibitor [5]. All cell types tested responded to this inhibitor *in vitro*, with an acceptable therapeutic index *in vivo* [5,40]. LAF389 underwent phase I clinical trials to treat high-grade solid tumours, but failed due to cardiovascular toxicity and inconsistent responses among patients [41]. The lack of data on target engagement in dose-response leaves open the question of whether these liabilities are off-target effects or due to dual inhibition of MetAP1 and MetAP2.

Concomitantly, Abbott (now AbbVie) reported the first rationally designed, reversible and selective MetAP2 inhibitor [6,17]. Based on a 2-amino-2-hydroxyamine core, the bestatin-like compound A-357300 **7** was shown to inhibit MetAP2 and the proliferation of human microvascular endothelial cells (HMVEC) [17]. It displayed anti-tumour activity in carcinoma, sarcoma and neuroblastoma murine models [17,42], and exerted weight-loss-related effects in rodents and primary brown fat adipocytes [18]. Abbott reported an anthranilic acid aryl sulfonamide series of reversible MetAP2i from mass spectrometry-based affinity selection screening, showing improved potency and selectivity over A-357300 [43,44]. Rational optimization led to A-800141 **8**, a reversible inhibitor that was more potent (IC_{50} = 12 nM, comparable to TNP-470 in their assay) and had oral activity [9]. Importantly, A-800141 showed anti-angiogenic and anti-cancer activity in a variety of tumour xenografts, including B cell lymphoma, neuroblastoma, prostate and colon carcinomas, after administration as single agent or in combination with other cytotoxic agents [45].

GlaxoSmithKline (GSK) identified a 1,2,4-triazole MetAP2i **9** through a high-throughput screen which potently inhibited endothelial cell proliferation and angiogenesis in an aortic ring tissue model in a dose-dependent manner [8].

Merck KGaA reported a series of MetAP2 reversible inhibitors based on a pyrimidine bicyclic core in 2017 [13]. Rational optimization of a high-throughput screening (HTS) hit led to the most potent compound **10** with IC_{50} 38 nM against MetAP2, while displaying favourable lipophilic ligand efficiency (LLE) [13]. However, HUVEC growth inhibition assays did not show the expected response and unable to optimize the structure of this series further, they turned to another HTS hit [14]. This compound, based on a cyclic tartronic diamide scaffold, was found to be active only after a spontaneous oxidation event during storage, and further isolation of the *S*-enantiomer and subsequent optimization steps led to compound **11** with potent anti-proliferative activity in HUVEC, favourable pharmacokinetic characteristics and robust anti-tumour activity in a murine glioblastoma model [14]. Efforts to prevent the enterohepatic circulation liability of **11** while maintaining potency led to clinical compound M8891 **12**, which is currently in phase I trials [15].

Second generation irreversible MetAP2 inhibitors for treatment of metabolic diseases

The fumagillin-derivative Beloranib **13** was initially developed by Chong Kun Dang Co. (CKD) Pharmaceuticals under the name CKD-732 and originally intended for the treatment of cancer [46]. It had undergone phase I clinical trials for the treatment of refractory solid tumours, either alone or in combination, with promising results when it was licensed to Zafgen in 2009 [47,48]. Despite its liabilities as a covalent inhibitor and intravenous mode of delivery, Zafgen further developed **13** as Beloranib (also known as ZGN-433) in phase I clinical trials for treatment of obesity [49], based on reports demonstrating the potential of irreversible MetAP2i, in particular CKD-732, to induce non-toxic weight loss [50–52]. Effects of **13** on adipose tissue and food intake occurred at lower doses than required for anti-tumour action and, according to the authors, vascularization was unaffected [49,51,52]. Confident of the safety profile at these lower doses, with adverse events of only mild nausea and dizziness [49], Beloranib was taken to phase II [53]. While impact on bodyweight loss and appetite reduction were promising and most common adverse effects were mild and sleep-related, three cases of venous thromboembolism caused the trial to be terminated early [53]. Despite these safety warnings, Zafgen pushed forward into phase III clinical trials for treatment of

Prader-Willi Syndrome, a rare genetic disorder leading to obsessive food intake and morbidity [54]. Unfortunately, two fatal events of pulmonary embolism and two events of deep-vein thrombosis occurred, halting the trial and forcing Zafgen to cease Beloranib's clinical development [54]. ZGN-1061 **14** was reported to have an improved safety profile over Beloranib, while maintaining its efficacy. According to Zafgen, this was due to reduced endothelial cell proliferation inhibition *in vitro*, achieved by lower intracellular concentrations, shorter half-life and reduced cellular MetAP2 inhibition by ZGN-1061 as compared with Beloranib [4]. With compelling results in preclinical studies, ZGN-1061 rapidly moved into phase I [55]. ZFN-1061 was put on hold by the Food and Drug Administration (FDA) while being tested in phase II trials in 2018, owing to cardiovascular safety risk (clinicaltrials.gov, ID: NCT03254368; https://zafgen.gcs-web.com/node/9456/pdf), and no further data have been reported.

Reversible MetAP2 inhibitors for the treatment of obesity

Combining a fragment-based drug discovery approach and optimization iterations based on structural studies, Takeda reported two series of reversible MetAP2i, exemplified by **15** (indazole) and **16** (pyrazolo[4,3-*b*]indole), showing significant and sustained weight loss in a mouse model of obesity when dosed orally [11,12]. Different parts of these lead compounds were combined to yield **17** [18,56]. Compound **17** reportedly led to sustained weight loss in obese rodents as well as to increased energy expenditure in human adipocytes derived from obese individuals [56]. Induced weight loss was caused by direct action of **17** on brown adipocytes [18], in contrast to previous hypotheses positing that weight loss activity was an indirect anti-angiogenesis effect in adipose tissue [57].

Reversible MetAP2 inhibitors for the treatment of autoimmunity

Recently, GSK described the first reversible indole MetAP2i series exemplified by carboxamide **18**, showing a dose-dependent effect on IgG production in differentiated memory B-cells in a mouse model of B-cell adaptive immunity [14,58].

Exploring the pharmacology and mode-of-action of MetAP2 inhibitors

Inhibition of cell proliferation and dependence on p53 signalling

Since the discovery of fumagillin's anti-angiogenic properties, many studies focused on the cytostatic effect of MetAP2i on endothelial cells. TNP-470 was shown to cause G0/G1 arrest and DNA synthesis inhibition [21], later confirmed for reversible MetAP2 inhibitor A-357300 [17], as did shRNA-mediated knockdown of MetAP2 in glioblastoma cells [59]. Despite the potential additional impact of MetAP2 knockdown on translation initiation (Box 1), together with previous studies these data support cell cycle arrest driving the anti-proliferative phenotype observed in certain cell lines. Cell cycle marker characterization did not seem to be fully consistent between studies, however: TNP-470 was reported to reduce cyclin D1 expression and inhibit retinoblastoma (RB) protein phosphorylation, a canonical marker of cell proliferation [60,61], whereas A-357300 did not alter cyclin D1 levels but instead dose-dependently decreased Cyclin A levels [17]. Covalent inhibitor PPI-2458 was shown to decrease the expression of another cell cycle component, proliferating cell nuclear antigen (PCNA), in a manner proportional to MetAP2 inhibition [62]. Interestingly, the cytostatic effect of TNP-470 was reported to persist after inhibitor washout at very high doses (670,000 × EC_{50}), while cells fully recovered at lower doses (ca. 670 × EC_{50}). Finally, actively proliferating cells displayed higher MetAP2 expression than serum-starved, arrested cells [63]. Together, these different lines of evidence support a role for MetAP2 in sustaining cellular proliferation through direct or indirect action on the cell cycle.

An early finding was a significant disparity in sensitivity between different cell types. While HUVECs were highly responsive, some other tumour cell lines needed doses at least 10-fold higher to exhibit a similar response [2,21]. MetAP2 has been shown to be essential in higher eukaryotes such as flies or mice [64,65] and has an important role in maintaining proliferation (Box 1), raising the question: how could some cell types show strong resistance to MetAP2i while others stalled efficiently at extremely low doses? Studies of MetAP2 levels in sensitive and resistant cell lines showed no significant difference at mRNA or protein levels [66,67], suggesting simple enzyme availability did not explain the observed cell selectivity by MetAP2i. As noted above, previous reports suggested that individual cells increased MetAP2 levels when actively proliferating and downregulated MetAP2 when arrested [63]; however, incubation of proliferating cells with fumagillin specifically increased MetAP2 levels in all cell lines tested, independently of the degree of antiproliferative response [66]. This perplexing cell line-dependence was later explained in a study showing that G1/G0 cell cycle arrest

exerted by TNP-470 was mediated by p53 and p21, and that cell lines lacking functional p53 or p21 were resistant [68].

Cytotoxicity

Fumagillin and its derivatives are cytotoxic at doses superior to those required for its cytostatic effects. TNP-470 showed complete inhibition of growth in HUVECs across a wide range of concentrations up to 200,000 × EC_{50} (EC_{50} = 30 pM) without apparent cytotoxicity and started becoming cytotoxic at doses >2×10^6 × EC_{50} (>75 µM) [21]. The cytotoxic effect was not cell-type specific [21], strongly suggesting off-target cytotoxicity unrelated to MetAP2 activity. A handful of studies also reported cell-type specific cytotoxicity. Fumagillin was shown to cause apoptosis in mesothelioma cells at a 1 µM dose, supposedly due to higher MetAP2 mRNA levels and therefore stronger dependency on this enzyme than their normal counterparts. This was linked to mitochondrial damage, nucleosome formation and caspase activation, and significant decreases in telomerase and anti-apoptotic Bcl-2 expression [23]. Treatment of cholangiocarcinoma cell lines with 6-50 µM TNP-470 was also reported to induce apoptosis (EC_{50} = 4 µM), this time through activation of p38-phosphorylation, up-regulation of Bax and down-regulation of Bcl-xL, as well as to enhance the anti-tumour activity of 5-fluorouracil, cisplatin, doxorubicin, and gemcitabine [25].

The underlying mechanism for cytotoxicity appears to be fundamentally distinct from the molecular pathways that drive the cytostatic effect. In addition to the differences in cell selectivity, the BCL2-family apoptosis regulator factors have not been reported in any publication describing cytostatic effects and the doses at which these are observed are consistently far higher than those reported for cytostatic activity. Moreover, it seems counterintuitive that agents that induce G0/1 arrest would synergize with broad spectrum chemotherapeutics [25] that primarily target highly proliferative cells.

Immune modulation

Fumagillin and its analogues, as well as ovalicin, show immunosuppressive properties [28–31] and both TNP-470 and PPI-2458 underwent clinical trials for the treatment of arthritis and joint inflammation [26,37,38,62]. In addition, MetAP2 expression was shown to be higher in germinal centre B cells, specifically in dark zone B cells, and corresponding cancerous cell lines, such as B-cell lymphoma but not in other non-B cell lymphomas such as Hodgkin's or T-

cell lymphomas [19]. A more recent study by GSK showed that MetAP2 inhibitor PPI-2458 efficiently blocked differentiation of B cells into plasma cells and subsequent antibody production [20]. Interestingly, this study also highlighted that the specific pattern of MetAP2 expression previously reported in human spleen and other lymph node samples [19] was species-specific and could be recapitulated in marmoset spleen samples but not in samples from mouse spleens [20]. This suggests species-specific differences in MetAP2 activities and poses challenges for translating observations from mice studies to human biology [20]. However, the most recent study by GSK studied the efficacy of **10** in a mouse-model of B-cell adaptive immunity, and showed that oral dosage with **10** in mice lead to reduced secondary antibody response [58].

Weight loss

The sustained weight loss effects of MetAP2i have been well known for many years. First reported as an undesired effect of TNP-470 in the 1990s [2], it took nearly 20 years until MetAP2i were specifically developed for the treatment of obesity and other metabolic disorders [11,12,49]. Most inhibitors reported to induce non-toxic weight loss have also been shown to inhibit endothelial cell proliferation [2,18,49,56], suggesting both effects depend on MetAP2 inhibition. For Beloranib, reduction of adipose tissue and food intake were reported to require lower doses than vasculature effects[49,51], suggesting differential systemic effects might depend on differential tissue distribution. Vasculature-related adverse events halted the development first of Beloranib, and then of ZFN-1061, the two lead candidates of Zafgen [53,54]. While weight-loss activity of MetAP2i was initially thought to be related to angiogenesis inhibition on adipose tissue [57,69], later evidence suggests otherwise, with A-357300, Beloranib and **17** shown to directly impact the metabolic signature of human primary adipocytes from obese individuals as well as brown adipocytes [18,56].

Embryonic development

The lab of Craig Crews, which had carried out important research on MetAP2 including solving the first structure of the complex with fumagillin [70], found that MetAP2-KO murine embryos fail to undergo gastrulation [65], and used a zebrafish embryo model to identify an important role for MetAP2 in early embryonic development. Treatment with TNP-470 or antisense MetAP2 oligonucleotide blocked the non-canonical Wnt/planar cell polarity (PCP) pathway

signalling in zebrafish embryos but did not affect the canonical Wnt/β -catenin pathway. They dissected that the effect of MetAP2 inhibition occurred downstream of the Wnt receptor Frizzled but upstream of calmodulin-dependent Kinase II (CamKII), RhoA small G-protein and c-Jun N-terminal kinase (JNK) [71]. Importantly, JNK activity has been linked to endothelial cell proliferation and both c-jun and RhoA are considered critical factors for angiogenesis [72,73]. The relationship between TNP-470 activity on non-canonical Wnt signalling and the cytostatic effect observed in certain cell lines was not clear. The same dose that inhibited Wnt signalling (10 nM) and blocked differentiation into primitive endoderm in a cell culture model exerted negligible anti-proliferative activity (less than 5%) [71]. In contrast, constitutive activation of the Wnt/PCP pathway seemed to slightly rescue the cell proliferation inhibition phenotype in mouse pulmonary endothelial cells by about 10-fold, but the full dose-response curves were not shown. A similar effect on HUVECs was mentioned, but data were not disclosed [71]. In a follow-up study, the authors further suggested that Wnt PCP pathway inhibition was driven by accumulation of Rab37, since induction of Rab37 accumulation alone by point-mutation led to the same aberrant phenotype as in MetAP2 inhibitor-treated embryos [74]. It must be noted this work was performed on overexpressed Rab37, since the authors were not able to detect endogenous Rab37 [74]. In an independent study, MetAP2 inhibition/knockdown was found to result in CamKII inhibition (part of the Wnt/PCP pathway) in zebrafish embryos and human umbilical cord blood (CB) models [75]. Interestingly, MetAP2 inhibition/knockdown also led to inhibition of definitive hematopoiesis, as well as increased ERK phosphorylation in developing vasculature [75]. A different study related MetAP2 inhibition by fumagillin to PI3K and AKT1 upregulation, which have also been linked to Wnt signalling [76], although according to the authors this was due to direct binding of fumagillin to fibroblast growth factor receptor 1 (FGFR1) [77]. Although care must be taken not to equate inhibition with knockdown/knockout studies due to the distinct roles of MetAP2 activity and scaffolding functions (Box 1), together these studies suggest a link of MetAP2 inhibition with non-canonical Wnt signalling and embryonic development in a manner not clearly related to the cell-cycle inhibition phenotype described for certain cell types. Furthermore, these phenotypes have not been reported in human patients dosed with MetAP2i, and therefore may be restricted to development with potentially limited relevance to treatment of adult patients.

In conclusion, a variety of phenotypic effects have been described for MetAP2i beyond the well-established inhibition of endothelial cell proliferation (Figure 3, Key Figure). The connections between the reported cellular and systemic phenotypes and the effects on specific signalling pathways remain poorly understood and warrant further investigation in order to progress selective and potent modulators of MetAP2.

Concluding remarks and future perspectives

MetAP2 has attracted significant interest as drug target in different diseases by major pharmaceutical companies over the years. Interestingly, nearly all MetAP2i that have reached clinical trials have been irreversible, fumagillin-like covalent MetAP2i, despite their evident liabilities [16,57]. These MetAP2i have subnanomolar potencies in endothelial cells and had promising results in preclinical models of cancer and obesity. However, fumagillin analogues possess one or two reactive epoxide groups that could lead to molecular instability and lack of specificity and could be the underlying reason for withdrawal from clinical trials due to adverse toxic effects. It is worth noting, however, that adverse effects have been diverse: while neurotoxicity was the main issue for TNP-470 [35], the obesity-targeting MetAP2i Beloranib and ZGN-1061 showed mainly cardiovascular toxicity [53,54]. No further reports from clinical development of PPI-2458 have been reported beyond the fact that it entered phase I (clinicaltrials.gov, ID: NCT00100347). Reversible MetAP2i arose as cleaner, more 'drug-like' alternatives with better pharmacokinetic properties than fumagillin derivatives. However reversible inhibitors were not considered potent enough to be of clinical value [78], since MetAP2i pharmacology appears to require a high degree of inhibition for an extended period. An exception was dual MetAP1/MetAP2 inhibitor bengamide LAF389 which did undergo phase I clinical studies. However, inhibiting both enzymes responsible for N-terminal methionine excision, which is an essential process in all organisms [79], was a risky approach, and this strategy was not pursued further. Recently, Merck KGaA published the first specific and reversible MetAP2 clinical candidate which entered phase I trials [15]; the future progress of this compound is a test case for the potential of reversible MetAP2i. Finally, it is interesting to note that the earliest examples of proteolysis targeting chimeras (PROTACs) were developed against MetAP2 [80]; novel applications may arise from next generation MetAP2-

targeted PROTACs since they would also target the scaffolding functions of MetAP2 and thereby phenocopy MetAP2 knockdown or knockout [81].

There remain major uncertainties regarding the mode-of-action of MetAP inhibitors (see Outstanding Questions). The different phenotypes of MetAP2 inhibition (anti-angiogenic and anti-tumour activities, immunomodulatory effect, and weight-loss) only seem loosely connected despite strong arguments in favour of a MetAP2-mediated mechanism in each case. Are the differential systemic effects driven by distinct tissue distribution of each compound? Whether weight loss is due to the antiangiogenic activity of MetAP2 on adipose tissues [57,69] or a direct effect on adipocytes themselves [18] is still unclear and some studies indicate independent mechanisms for each effect [49,51,52]. It will be necessary to understand the link between these different activities, however, to develop pharmacological compounds with a specific effect, which is important since a desired effect in a specific indication, e.g. weight loss in obese patients, might become adverse events in another, e.g. weight loss in patients with advanced solid malignancies. Some studies seem to indicate a single selective effect might be achievable for MetAP2i, as it seems MetAP2i designed for the treatment of cancer largely avoid the weight-loss effect in mice [2,14]. Unfortunately, pre-clinical studies are often not translated in humans [82]. Clear differences were found in MetAP2 expression patterns in human vs mouse spleen tissue samples [20], suggesting that the adaptive immune suppression effect observed for some MetAP2i might be overlooked in mouse models of other diseases. Moreover, whether an anti-obesity phenotype can be obtained without antiangiogenic effects remains to be seen; notably, major adverse effects in Zafgen's trials of irreversible MetAP2i relate to deep vein thromboembolism events [53,54].

The specific molecular changes induced by MetAP2 inhibition at the cellular level remain unclear. It is currently unknown whether the G0/G1 cell cycle arrest relates to the effect seen in the Wnt/PCP pathway, and how this might drive cancer-specific targeting in solid tumours. Even less clear are the molecular changes that lead to systemic weight-loss. Further, how MetAP2 might drive all these responses and which MetAP2 substrates drive these effects is yet to be discovered. The specific substrates of each MetAP isoform are still not clear (Box 1, [83–85]), thereby hindering the elucidation of MetAP2i mode-of-action. Moreover, iMet removal by MetAPs precedes most other N-terminal modifications (e.g. *N*-acetylation [83], *N*-myristoylation [86], etc.) as well as other important processes regulating proteome dynamics

[87,88] (Box 1), which implies MetAP2i effects could be mediated by the influence of MetAP2 on downstream protein regulatory and processing events that impact the function of one or a subset of proteins. Studies that reveal a comprehensive list of MetAP2-specifc substrates that are directly affected upon MetAP2 inhibition will be invaluable to start answering these questions. Importantly, substrate elucidation and mode-of-action studies have the potential to increase success rates of clinical MetAP2i candidates at three different levels: (1) they can lead to the discovery of novel and better biomarkers, which can not only provide improved diagnostics but also support patient selection in clinical trials; (2) they can suggest new indications for therapeutic applications in which MetAP2i might be more useful than in any of those previously explored; and (3) they might suggest new combination treatment options through prediction of synergistic or additive effects, which are important for oncology indications and one of the main areas of development for MetAP2i (see above). A better understanding of the molecular mechanism of MetAP2i is likely to be the key to finally deliver a drug from three decades of investment in MetAP2i discovery and development.

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Boxes and figure legends

Box 1 (& Figure 1). Biological role of MetAP2

In humans, MetAP2 has been shown to have two distinct biological functions, both implicated in protein synthesis regulation. The enzymatic function of MetAP2 is responsible for initiator methionine (iMet) removal, a role shared with MetAP1 [83]. This process has been estimated to occur in >70% of proteins synthesised in the cell and it is one of the first modifications a protein may undergo [83]. It is assumed to be primarily a co-translational modification, where iMet removal precedes most other N-terminal modifications [83] as well as most other processing events such as protein folding or organellar targeting [88], and regulates protein degradation through N-degron pathways [87] thus playing an important role in maintaining protein homeostasis. Both human MetAPs have very similar substrate sequence preferences in the first few amino acids, and to date neither the cellular substrate profiles nor the relative contribution of each MetAP to total cellular MetAP catalytic activity have been defined [84,85,89,90]. However, whilst iMet excision is an essential process in development the subtle and well-tolerated effects of MetAP2i strongly suggest that there is redundancy between MetAPs in essential cellular pathways in adults. The second reported role of MetAP2 is nonenzymatic, whereby its N-terminal polycharged domain binds eukaryotic initiation factor 2 alpha (eIF2α, also known as EIF2S1) and protects it from inhibitory phosphorylation, promoting general protein synthesis in a manner independent from the catalytic domain [91]. Finally, MetAP2 has been reported to possess autoproteolytic activity resulting in two stable fragments: an N-terminal 26 kDa fragment and a C-terminal 52 kDa fragment, separating the protection of eIF2α phosphorylation and aminopeptidase functions, respectively [92]. Importantly, both covalent and reversible MetAP2 inhibitors have been reported to affect aminopeptidase activity only, without interfering with the binding to eIF2α [28].

(Inside Box1) **Figure 1. MetAP2 has two distinct functional roles, mediated by two different protein domains.** MetAP2 cleaves iMet from nascent peptides, carried out by the catalytic domain, whilst the N-terminal polycharged domain binds eIF2α, protecting it from inhibitory phosphorylation by eIF2K and promoting translation.

Figure 2. Prevalent covalent and reversible MetAP2 inhibitors. The natural compounds fumagillin (**1**) and ovalicin (**3**), the semi-synthetic analogues TNP-470 (**2**) [2] and PPI-2458 (**4**) [10], and the more drug-like spiroepoxytriazole (**5**) [39]. The dual MetAP1/MetAP2 reversible inhibitor LAF389 (**6**) [5], A-357300 bestatin-like compound (**7**) [6], anthranilic acid sulfonamide A-800141 (**8**) [9], triazole (**9**) [8], triazolo pyrimidine (**10**) [13], cyclic tartronic acid (**11**) [14] and M8891 clinical compound from Merck KGaA (**12**) [15]. Second generation fumagillin derivatives reported for treatment of obesity and metabolic diseases, Beloranib (also known as CKD-732 or ZGN-433) (**13**) [46] and ZGN-1061 (**14**) [4]. Reversible MetAP2 inhibitors targeting obesity: indazole (**15**) [11], pyrazolo[4,3-b]indole (**16**) [12] and pyrimido(pyrazolo[4,3-b]indole) (**17**) [18]; and autoimmunity: 2229238A carboxamide (**18**) [58].

Figure 3 (Key Figure). MetAP2 inhibition results in distinct cellular effects and phenotypes. These include the cytostatic effect, which is the most studied activity of MetAP2i and is responsible for the characteristic anti-angiogenic activity due to inhibition of endothelial cell proliferation; non-toxic weight loss and immunomodulatory effects, exploited in the development of specific inhibitors; and less studied developmental and potentially off-target cytotoxic activities.

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Figure1

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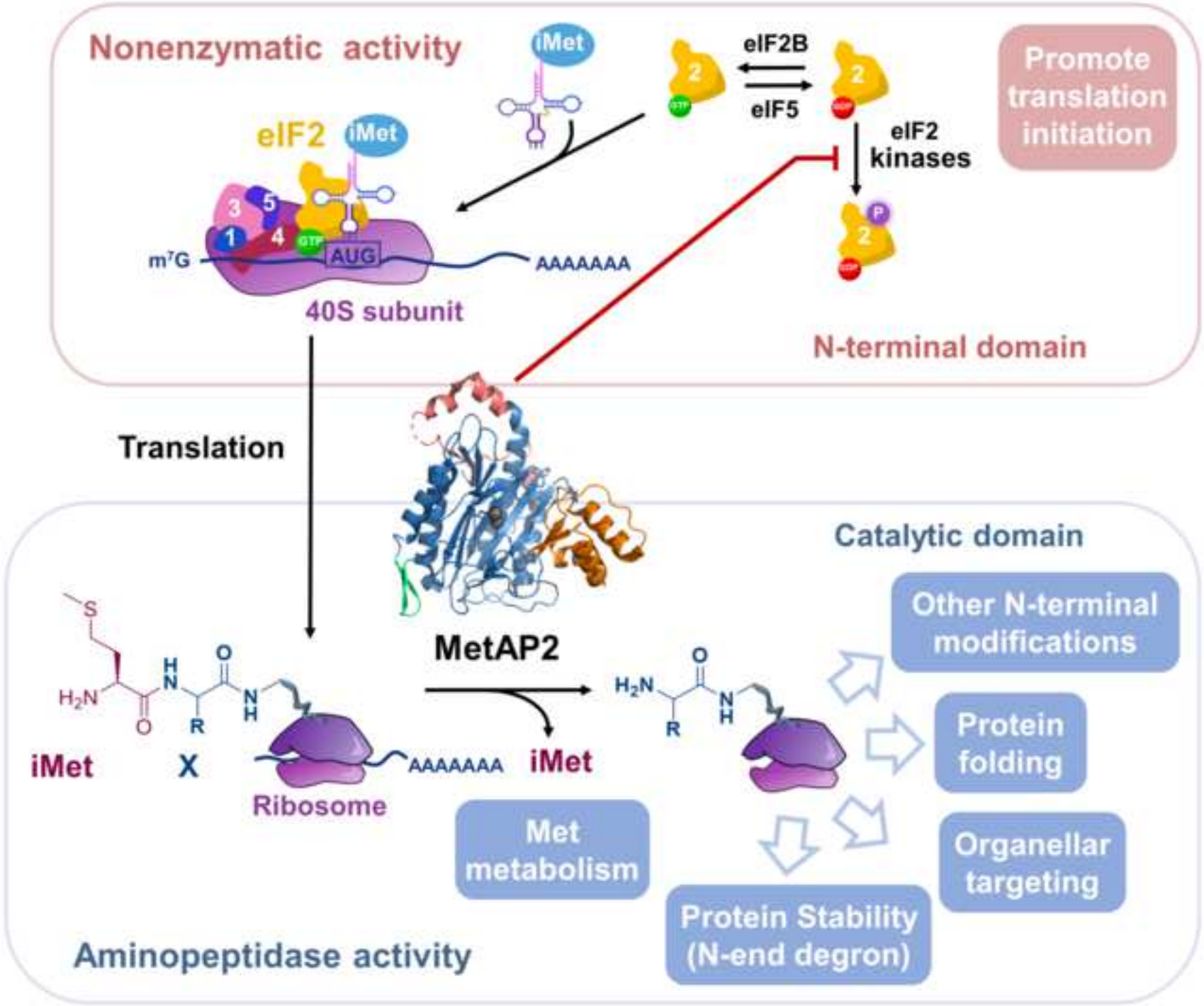

Figure 2

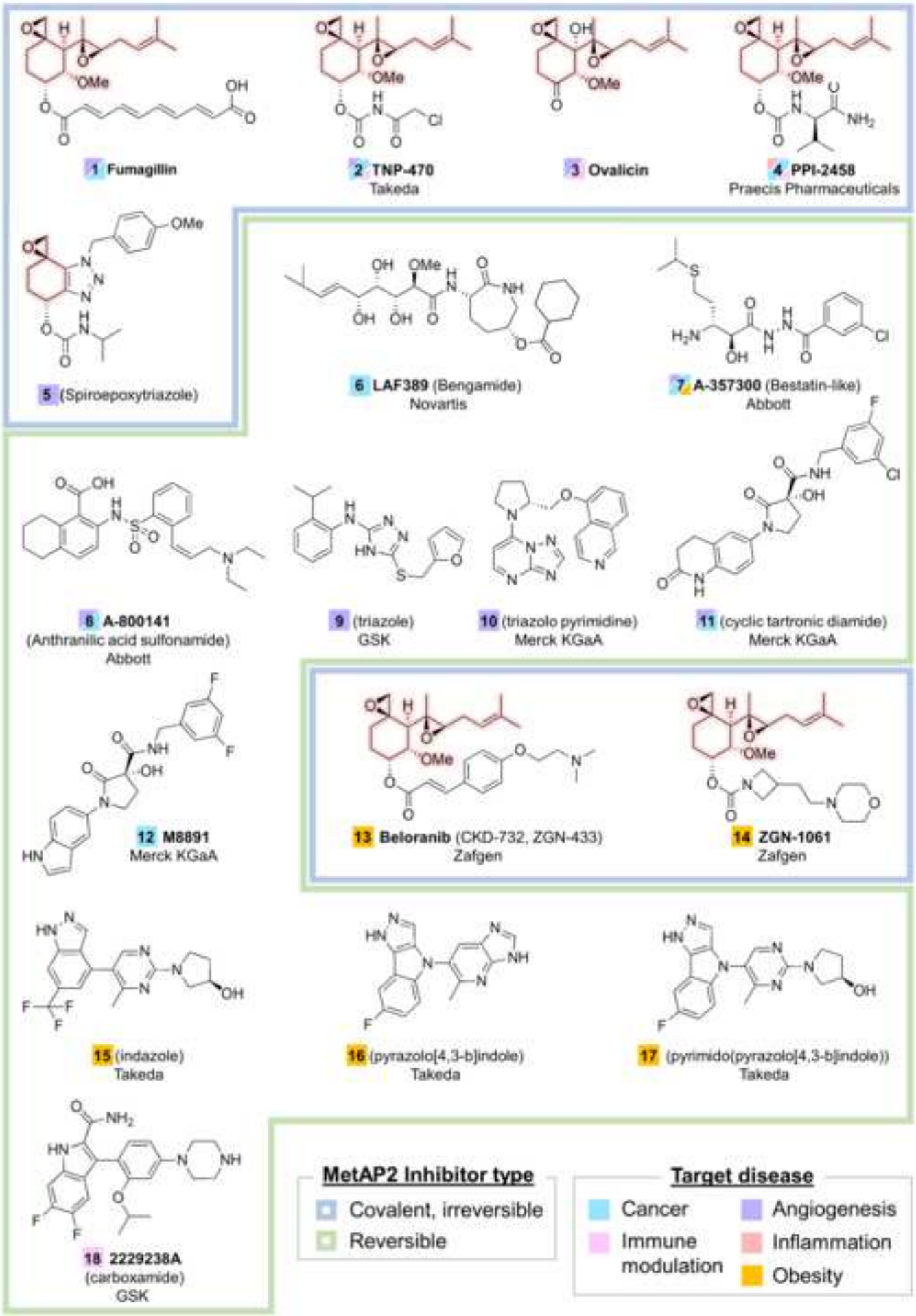

Figure 3 (Key Figure)

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