Molecular Pathways: Preclinical Models and Clinical Trials with Metformin in Breast Cancer

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ABSTRACT

Metformin, an oral biguanide widely used to treat diabetes, has considerable potential and is in clinical trials as an experimental preventive or therapeutic agent for a range of cancers. Direct actions targeting cellular pathways, particularly via AMPK and through inhibiting mitochondrial ATP synthesis, or systemic mechanisms involving insulin and insulin like growth factors have been much studied in vitro and in preclinical models. Epidemiological and retrospective studies also provide clinical evidence in support of metformin as an antitumor agent. Pre-operative window of opportunity trials confirm the safety of metformin in women with primary breast cancer, demonstrate reduction in tumor cell proliferation and complex pathways of gene suppression or overexpression attributable to metformin. Confirmation of insulin mediated effects, independent of body mass index, also supports the potential benefit of adjuvant metformin therapy. Neoadjuvant, adjuvant and advanced disease trials combining metformin with established anticancer agents are underway or proposed. Companion biomarker studies will utilise in vitro and preclinical understanding of the relevant molecular pathways to, in future, refine patient and tumor selection for metformin therapy.
BACKGROUND

Synthetic biguanides, based on the guanidine derivative galegine originally derived from Galega officinalis (French lilac), have been used clinically since the 1950s with metformin (N’,N’-dimethylbiguanide) prescribed to over 120 million type II diabetics globally. Phenformin, previously used in the treatment of diabetes, was withdrawn from many countries in the 1970s due to drug associated lactic acidosis (1). The potential for re-purposing metformin to oncology is supported by evidence of reduced cancer incidence and increased time to develop cancer (2), reduced mortality from cancer (3), with lower HER2 positive breast cancer specific mortality (4) or reduced risk of distant metastases in triple negative breast cancer (5) in diabetic women. Additional supportive evidence has come from studies demonstrating higher serum insulin levels are associated with increased risk of breast cancer (6) and shorter survival from breast cancer (7) which may be modified by metformin.

The relevance of the multiple proposed mechanisms of action of biguanides to cancer remain controversial. In vitro, in vivo animal model and human studies support several possible modes of action with extrapolation of studies in diabetes, the range of in vitro models, non-physiological metformin levels, the circumstantial evidence from epidemiological studies and retrospective reviews creating complexity. Focussed initially on diabetes and metabolism more generally, both direct (cellular) pathways and indirect (systemic) actions have been proposed as key mechanisms for the action of biguanides in diabetes that may also be of relevance to cancer.

AMPK mediated effects.

The master sensor of cellular energy, the Ser/Thr protein kinase complex AMP activated protein kinase (AMPK) remains central to studies of metformin. AMPK activation stimulates TSC2, hence indirectly as well as AMPK directly inhibits mTOR and consequently by down regulating S6kinase leads to reduced protein synthesis, reduced cellular growth and lowers proliferation (Figure 1). A
complex containing Liver kinase B1 (LKB1), classed as a tumor suppressor gene, activates heterodimers of AMPK subunits through phosphorylation of AMPKa2Thr-172, established as a link between LKB1, AMPK and cancer (8). Breast cancer cells lacking LKB1 are resistant to the growth inhibitory effects of metformin in vitro and in vivo (9,10,11), and as a distinct mechanism, epigenetic inactivation of LKB1 has been reported in sporadic papillary breast cancer (12). Furthermore, the intracellular pathways down stream of HER2 amplification, PIK3CA mutation and PTEN mutation or loss of PTEN expression converge on elevated mTOR signalling and are considered potential mechanisms for breast cancer development and of resistance to therapy. This makes the potential for metformin to work via targeting the mTOR cascade an attractive mechanism of action (13).

Other, AMPK and TSC2 independent, mechanisms of mTOR inhibition by metformin, include suppression of RAG GTPases (14).

Additional, potentially important, AMPK mechanisms for oncology include phosphorylating and hence inactivating ACC1 and ACC2, consequently blocking fatty acid synthesis but increasing mitochondrial fatty acid uptake and subsequent breakdown by β-oxidation (15). Inactivation of AMPK promotes a metabolic shift to aerobic glycolysis through stabilisation of HIF1a, increases transcription of genes involved in glycolytic pathways, resistance to apoptosis, angiogenesis and metabolic adaptation (16,17). AMPK may act as a negative regulator of the Warburg effect under nutrient rich and growth factor replete conditions in the absence of evident energy imbalance (16). In contrast, AMPK activation may increase resistance to stress and viability of cancer cells under hypoxic conditions of metabolic stress (18) which may be of particular relevance as tumor cancer cells migrate and metastasise.

AMPK phosphorylates p53 and leads to cell cycle checkpoint activation, at least in mouse embryonic fibroblasts, allowing cells to survive energy deprivation (19). Indeed, metformin inhibition of complex 1 of the mitochondrial electron transport chain (20,21,22) is particularly effective in p53-deficient colon cancer cells (23) although the p53 status did not affect metformin sensitivity in other
cancer cell lines (24,25). However, loss of functional AMPK or p53 (as is common through mutation or p53 network dysfunction in most cancers) may constitute a sufficient energy crisis to result in metformin having a cytotoxic synthetic lethality effect (26). Thus, there is evidence that metformin may be therapeutically effective acting via p53 particularly where normal p53 function is abrogated, as is the case in most epithelial cancers.

**Mitochondrial effects**

Biguanides accumulate in mitochondria, with uptake promoted (for the cationic metformin at least) by organic cation transporter 1 (OCT1) to inhibit Complex I of the mitochondrial respiratory chain, inhibit mitochondrial ATP synthesis, increase ADP:ATP and AMP:ATP ratios and hence activate AMPK indirectly. Phenformin is 50 fold more potent than metformin as an inhibitor of mitochondrial complex 1 and being lipophilic (21) is less dependent on OCT (27) retaining the dose-dependent antiproliferative effect in ovarian cancer cell lines even in the presence of OCT1 siRNA (28). Given that oxidative phosphorylation-dependent production of ATP appears to be secondary to mitochondrial synthesis of anabolic precursors in proliferating cells (29), it has been suggested that metformin exerts the chemopreventive effects by functioning as a weak mitochondrial poison inhibiting oxidative phosphorylation (30).

Additional, non-AMPK mediated mechanisms for the antineoplastic effect of metformin have been proposed through NF-κB, a pleiotropic protein complex that controls DNA transcription. Metformin may inhibit IκB and IκKα/b subunits and hence NF-κB and prevent the translocation of NF-κB from the cytoplasm to the nucleus (31). Metformin appears to down regulate genes involved in mitosis and may act synergistically in this setting with taxanes (32). Metformin may also act via the DNA damage response mechanisms through ataxia telangiectasia mutated (ATM) and Chk2 (33), also potentially accounting for the cancer prevention effect.

**Systemic effects**
Biguanides have long been known to suppress hepatic glucose production (34) with metformin uptake via OCT leading to AMKP inhibition of hepatic gluconeogenesis, increase peripheral insulin sensitivity (and hence glucose uptake for example in skeletal muscle) and reduce circulating insulin and IGF-1 levels, although it is less clear how metformin regulates IGF-1.

Certainly, the insulin receptor lies upstream of the PI3K/AKT/mTOR signalling pathways and breast cancer cells may have greater sensitivity to the effects of insulin such that metformin reducing insulin levels and hence binding of insulin to receptors on breast cancer cells present very attractive mechanisms to indirectly inhibit tumour growth (13). This provides molecular insights into and supports the clinical relevance of insulin/IGF-1 reduction in relation to cancer progression (7,35,36).

CLINICAL-TRANSLATIONAL ADVANCES

Phenformin

In murine models, phenformin has been associated with a reduced incidence of DMBA induced rat mammary tumors (37), enhances the effects of chemotherapy (38) and elicits antiproliferative effects, alongside inducing apoptosis, in neuroblastoma (39). Phenformin, like metformin, is associated with enhanced apoptosis and ATP depletion in AMPK deficient cancer cells and may be most effective in cancer cells without a functional LKB1-AMPK pathway, as demonstrated in non-small cell lung cancer (40).

Phenformin has been shown to prevent and retard growth of breast cancer cell lines(41) and to protect against tumour development in a spontaneous lymphoma murine model exhibiting PTEN heterozygous mutations and LKB1 hypomorphic mutations with greater potency than metformin (11).

Mechanistic insights from xenograft work suggest both systemic and direct, tumor/stromal effects may be elicited (41)and has led to speculation that phenformin may have the capability to block a
reverse Warburg effect of onconutrient production by stroma feeding breast cancer cells in vivo (42,43).

Phenformin may initially seem most attractive for trials in the advanced breast cancer setting (providing renal and hepatic function are adequate) with the potential to avoid lactic acidosis (1) and potentially enhance cancer cell kill by concommitant administration of 2 deoxyglucose with phenformin (if confirmed beyond colorectal cancer cell lines) (44).

Metformin

In vitro models suggest metformin may be active against breast cancer cell lines regardless of the estrogen receptor (ER), progesterone receptor (PR), HER2 status or p53 status and subtype of the cells (24,25).

For ER positive breast cancer, dependent on aromatase conversion of precursors to estrogens in postmenopausal women, metformin may inhibit aromatase enzyme expression in breast adipose tissue via AMPK (45). Similar effects on other clinically relevant genes have yet to be explored. Given that aromatase inhibitors (AI) are now the mainstay of neoadjuvant, adjuvant and extended treatment in postmenopausal women with breast cancer, the potential for synergism between an AI and metformin is under exploration in a Korean neoadjuvant trial and is planned for an international extended adjuvant trial. For premenopausal women, where tamoxifen remains the principal adjuvant therapy for ER positive breast cancer, the additive effects of metformin to tamoxifen, at least in terms of reducing cell proliferation (46), merits subgroup analysis within the ongoing MA32 adjuvant metformin trial (47).

For HER2, breast cancer lines expressing HER2 have increased sensitivity to metformin (48) mediated via inhibition of S6K; in trastuzumab resistant models, metformin disrupted ERBB2/IGF1R complexes and significantly reduced cell proliferation (49). Metformin, used at comparable clinical doses, delayed the onset, reduced the growth and increased the lifespan of MMTV-her2/neu mice (50).
accompanied by reductions in circulating insulin levels. As HER2 targeted therapy extends to other tumor types (such as gastric and oesophageal cancer) similar metformin therapy approaches may merit consideration.

For triple negative breast cancer, metformin was effective in vitro (and in an animal model) inducing AMPK and suppressing EGFR, MAPK and Src phosphorylation and lowering Cyclins D1 and Cyclin E (51). The effect of metformin on triple negative cell lines may, in part, be attributable to STAT3 inhibition (52).

More generally, reduced immunohistochemical staining for pAMPK was described in 318/349 (91%) breast cancers, with all subtypes of breast cancer represented (53). Whereas AMPK appears to protect against initial tumour development, in established breast cancers lack of functional AMPK dependent pathways may render breast cancer susceptible to metformin therapy.

Additional support for metformin as a therapeutic agent comes from in vivo models where metformin appears to selectively inhibit CD44+/CD24low cancer stem cell like populations (54), repress genes associated with epithelial mesenchymal transition such as ZEB1 TWIST, SNAi1 (55) and work synergistically with chemotherapy agents (54). Additionally, inhibition of glutamine metabolism, at least in prostate models, may synergise with metformin (56). Injected into tumours, albeit at potentially non-physiological doses, metformin may be effective alongside paclitaxel and carboplatinum as part of a combination chemotherapy strategy for the treatment of breast cancer (57).

However, some have questioned the relevance of selected preclinical data given the concentrations, doses and delivery of metformin employed (58). Nevertheless, studies have demonstrated cell inhibition using physiologically possible concentrations of metformin in vitro (51) and in mouse models (41,50,59). Even so, the multiplicity of mechanisms deduced in vitro may differ significantly
in vivo while animal models (usually in immune compromised mice) may be different again from the
cancer prevention and cancer therapy settings in humans.

**Metformin and breast cancer trials**

Given the preclinical data and that metformin has known pharmacokinetics and manageable
toxicities, with extensive use worldwide in diabetes, re-purposing metformin within clinical trials to
seek efficacy against breast cancer is very attractive. Evidence to date suggests metformin could be
clinically useful in the prevention, preoperative, adjuvant, extended adjuvant and advanced disease
settings, although the detail of dose, duration and drug combinations will require elucidation.
Fortunately, the research community is actively engaged to address many of these roles and through
companion biomarker studies ascertain the tumor and patient characteristics most amenable to
treatment and whether direct or systemic effects are both of clinical importance.

Trials are underway examining these issues in the setting of breast, brain, colorectal endometrium,
head and neck, lung, lymphoma, melanoma, ovary, pancreas, prostate, renal and thyroid
malignancy. Single centre, phase II, biomarker studies and/or with clinical endpoints predominate;
many trials use gradual increases in dose upto 1500-2250mg metformin per day to mitigate the self-
limiting initial gastrointestinal side effects of nausea, diarrhoea and bloating. Caution over the
potential but rare metformin associated lactic acidosis (1/100,000 years of use) generally restricts
the clinical trials populations to women under 75 years with good renal and hepatic function.

A proposed study of metformin versus placebo to prevent the progression of atypical ductal
hyperplasia, ductal or lobular in situ neoplasias appears attractive given evidence that even low dose
metformin (250mg/day) is sufficient to reduce proliferation and aberrant crypt foci in rectal
epithelium (60). The progression from DCIS to invasive breast cancer may be mechanistically linked
to glycolytic cancer associated fibroblast activity (exporting lactate to the tumour cells as evidenced by a fall in caveolin and rise in MCT4 expression) with the potential for blockade by metformin (61).

The first randomised clinical trial using metformin (1000mg bd) in breast cancer was reported in 2011 and used the preoperative window of opportunity setting (62). This novel trial randomised patients to receive metformin for two weeks or watchful waiting for two weeks between diagnostic biopsy and definitive surgery. Core needle tumour biopsies were taken at the time of diagnosis and on the operating table prior to resection of the operable breast cancer to allow direct comparison of tumour proliferation (Ki67), tumour messenger RNA expression alongside serum insulin. The trial demonstrated a significant reduction in Ki 67 with metformin but not in the control arm, reduced gene expression (including in the PI3 kinase pathway) and increased gene expression (for example of TNFR1), with a flattening of the serum insulin levels in response to surgery for the patients on metformin. Thus, both in vivo anti-tumour effects and systemic effects were demonstrated for the first time in women with primary breast cancer receiving metformin. The insulin effect, although women were not fasting at the time of diagnostic biopsy, and the lack of association between metformin effects and body mass index confirms the potentially beneficial systemic effect of metformin as an insulin suppressor in women with breast cancer (7,63).

A second, single centre, preoperative window of opportunity placebo controlled trial of similar size confirmed the reduction in Ki 67 with metformin use over two weeks, again not associated with BMI, and provided enhanced evidence for the systemic insulin mediated effects (64). The need for meticulous trial design was flagged by a subsequent trial (65) where failure to continue metformin up to the time of surgery and using core samples at diagnosis versus resection specimens (66) make data interpretation difficult.

Overall, these prospective, preoperative window studies in breast cancer (62,64,65), despite reservations regarding trial design and methodology, demonstrate metformin is safe for women with primary breast cancer and confirms the attraction of metformin as a therapeutic agent.
Given the proposed mechanisms of action of metformin and the preclinical data, synergism of the biguanide with chemotherapy has been explored. In a retrospective case note review of breast cancer patients receiving neoadjuvant chemotherapy (67) demonstrated a remarkable 24% complete pathological response (cPR) in diabetic patients incidentally taking metformin and receiving neoadjuvant chemotherapy compared with 16% cPR in nondiabetics and 8% cPR in diabetics not receiving metformin. A similar, also historical, series of epithelial ovarian cancer has demonstrated clinical benefit with increased progression free survival and overall survival in diabetics taking metformin receiving taxane/ carboplatin chemotherapy compared with nondiabetics or diabetics not on metformin (68). However, incidental metformin use has not demonstrated survival benefit in two case series of diabetics with breast cancer (5,67). This concept of combining metformin with neoadjuvant chemotherapy is in prospective trials of metformin along side paclitaxel and FAC; docetaxel, epirubicin and cyclophosphamide +/- metformin; and in ISPY2 (ganitumab + metformin). An innovative trial being conducted in Oxford combines the window of opportunity and neoadjuvant concepts. The Oxford trial sequences a two-week run in with metformin, then neoadjuvant chemotherapy (+/- metformin) is administered up to the time of surgery. Non-invasive PET scanning and lipid metabolism studies are being used alongside tissue biopsies to examine the effects of metformin on breast cancer metabolism and lipid synthesis.

In addition to the potentially synergistic effects of metformin with chemotherapy against breast cancer, murine models suggest a cardio protective effect of metformin which appears to abrogate the effects of doxorubicin on LDH, CKMB and glutathione (69), although evidence for such effects in humans is, as yet, lacking.

In the adjuvant setting, the NCIC CTG MA.32 trial (47) is examining the efficacy of adjuvant metformin for five years after complete resection of invasive breast cancer and following completion of chemotherapy and/or radiotherapy but concomitant with endocrine therapy or anti-HER2 therapy. Early data suggest beneficial effects of metformin compared with placebo on patient
weight and metabolic factors which, if sustained, could significantly improve survival from breast cancer (7). Further clinical efficacy, companion biomarker and mammographic density studies are eagerly awaited as the trial matures. There are also plans underway to trial metformin in the extended adjuvant setting, beyond five years, for ER positive patients at risk of recurrence. Further survivorship trials employing metformin along with exercise in breast and colon cancer are proposed.

In contrast to most clinical drug development, where molecular pathways are usually explored in advanced disease, this setting has only recently been reported for metformin. For ER+ advanced disease, there is no disadvantage to taking metformin concomitant with exemestane on exemestane levels (70) providing reassurance for the neoadjuvant and adjuvant trails using concomitant metformin and endocrine therapy underway. Interactions of metformin with a range of agents are proposed, based on an appreciation of the intracellular pathways outlined above, including trials of metformin with everolimus and exemestane in ER+ disease, metformin with erlotinib in triple negative breast cancer and combination chemotherapy +/- metformin in HER2 negative advanced disease.

Biomarker studies built in to all these trials will be essential to identify the breast cancers with characteristics (such as LKB1-AMPK or p53 defective pathways) most likely to be susceptible to the effects of metformin. Cancer heterogeneity of OCT1, and hence for cancer cell uptake of metformin, as observed in ovarian cancer (28), may merit measurement before considering metformin therapy in the clinical setting given, for example, the low expression of OCT1 in normal breast tissues. The potential effects of polymorphisms of LKB1, OCT 1,2,3 and ATM on resistance to metformin (70) and interactions between proton pump inhibitors and metformin via OCT 1,2,3 (70) will clearly need to be considered when metformin translates into routine clinical practice.
Biguanides, and particularly metformin, present an attractive proposition for repurposing as low toxicity anti-tumor agents, thus demonstrating how understanding the molecular pathways of cancer combined with clinical data will lead to further advances in oncology.

References


Metformin (M), absorbed from the gut, acts via systemic and intracellular molecular pathways. Systemic actions of metformin via metformin uptake through OCT in the liver, AMPK inhibition of gluconeogenesis and consequent reduction in insulin reduces insulin receptor (IR) stimulation and downstream intracellular effects (including via PI3kinase, akt and HIF1a) and leads to increased glucose uptake in tissues such as skeletal muscle. Cell level molecular pathways include reductions in mitochondrial respiratory function, activation by metformin of AMPK with some of the consequent intracellular events converging with the systemically driven insulin receptor pathway. AMPK stimulates phosphorylation of p53, ACC1 and ACC2, inhibits aromatase enzymes, directly and indirectly inhibits mTOR itself stimulated by key drivers for cancer (HER2, PIK3CA, PTEN) and hence curtails S6kinase activity and its consequences. Additional potentially important effects, for example on the stroma and the complexities of subcellular networks are not illustrated.
Figure 1:
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