REVIEW

Metformin in cancer: translational challenges

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Abstract

The anti-diabetic drug metformin is rapidly emerging as a potential anti-cancer agent. Metformin, effective in treating type 2 diabetes and the insulin resistance syndromes, improves insulin resistance by reducing hepatic gluconeogenesis and by enhancing glucose uptake by skeletal muscle. Epidemiological studies have consistently associated metformin use with decreased cancer incidence and cancer-related mortality. Furthermore, numerous preclinical and clinical studies have demonstrated anti-cancer effects of metformin, leading to an explosion of interest in evaluating this agent in human cancer. The effects of metformin on circulating insulin levels indicate a potential efficacy towards cancers associated with hyperinsulinaemia; however, metformin may also directly inhibit tumour growth. In this review, we describe the mechanism of action of metformin and summarise the epidemiological, clinical and preclinical evidence supporting a role for metformin in the treatment of cancer. In addition, the challenges associated with translating preclinical results into therapeutic benefit in the clinical setting will be discussed.

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Introduction

Emerging evidence from several areas of research suggests that metformin, a commonly used anti-diabetic drug, may be useful in the prevention and treatment of cancer. Metformin, a biguanide derivative, has been used for half a century to treat non-insulin-requiring diabetes. It is a relatively safe drug, with known pharmacokinetics and manageable toxicities. Its most common toxicity is mild-to-moderate gastrointestinal discomfort, usually self-limited and ameliorated by a graduated ramp up in dose. Its most serious toxicity, lactic acidosis, is rare (Bodmer *et al.* 2008), occurring once per 100 000 years of use. Lactic acidosis is more common in elderly patients (over age 80 years) and in those with impaired renal, cardiac and/or hepatic function. Although metformin has only recently been directly tested in conjunction with anti-cancer agents in the clinical setting, its common use in the treatment of diabetes has resulted in its co-administration with virtually all anti-cancer agents in diabetic cancer patients. No evidence of important interactions of metformin with standard cancer therapies has emerged through this experience. Because of this, clinical evaluation of metformin as an anti-cancer agent has

bypassed the traditional Phase I pharmacokinetic/toxicity assessment and has moved directly into Phase II and Phase III testing, reflecting rapid accumulation of preclinical, clinical and epidemiological evidence that it may have beneficial anti-cancer effects, despite the fact that precise mechanisms of potential anti-cancer action remain unresolved (Goodwin *et al.* 2011).

Current understanding of potential anti-cancer effects of metformin raises the intriguing possibility of a duality of action – metformin may act directly on the tumour or it may act indirectly on the host by lowering insulin levels. This, along with the fact that the key host-related mediator (insulin) is a critical component of culture medium used in *in vitro* experiments, leads to enhanced challenges in translating results of preclinical research into the clinical setting, underscoring the importance of cross-disciplinary research approaches.

Here, we summarise emerging evidence from research on humans (epidemiological, clinical and metabolic) and from the preclinical setting (mechanistic, *in vitro* and *in vivo* research) suggesting that metformin has anti-cancer effects (Fig. 1). We then integrate this information to develop recommendations for translational evaluation of metformin in the clinical setting.

Figure 1 Evidence supporting the use of metformin in the treatment of cancer. The potential use of metformin in the prevention and treatment of cancer is based on emerging evidence from a number of research fields. Metabolic factors such as obesity and hyperinsulinaemia are associated with cancer risk while epidemiological studies have demonstrated a reduced incidence of cancer in diabetic patients receiving metformin. Moreover, preclinical studies have provided evidence of the anti-cancer effects of metformin and defined its mechanism of action.

Research on humans

Epidemiologic

Evans *et al.* (2005) were the first to report a potential association of metformin use with reduced cancer incidence. In diabetics receiving metformin (as opposed to other therapies), overall cancer incidence was lowered (odds ratio (OR) 0.86 and 95% confidence interval (95% CI) 0.73–1.02) and there was evidence of a dose response in relation to total duration of use or number of prescriptions dispensed. Since then, at least 17 epidemiological studies examining the association of metformin treatment of diabetes with cancer incidence and mortality have reported similar findings (see Table 1), and evidence has emerged that metformin use may reverse the increased cancer risk associated with administration of insulin or insulin secretagogues (Li *et al.* 2009). A recent meta-analysis (Decensi *et al.* 2010) has identified a 31% reduction in overall cancer incidence or mortality when metformin is used in the treatment of diabetes. There is emerging evidence that metformin use is associated with reduced risk of several common cancers, including colorectal, pancreas, hepatocellular, breast and lung, although not all studies have identified significant effects. The reduced risk of obesity-associated cancers such as breast and colorectal is not surprising, given that metformin reduces insulin levels that appear to be important mediators of associations of obesity with cancer. Preclinical reports suggesting reduced tobacco-carcinogen-induced lung cancer burden with the use

of metformin (Memmott *et al.* 2010), coupled with emerging clinical evidence that metformin may be associated with improved lung cancer outcomes (reviewed below Mazzone *et al.* (2010) and Tan *et al.* (2011)), suggest that other factors, such as nicotine, may lead to insulin resistance in individuals with smoking associated cancers or that non-insulin-mediated mechanisms of action are important.

Although epidemiological studies have fairly consistently reported reduced cancer incidence and/or mortality in diabetic patients who receive metformin in standard clinical doses (1500–2250 mg/day in adults), these studies have important methodological limitations. Most were conducted retrospectively and many sampled their cases from hospital or clinical registries rather than population-based registries, thereby limiting external validity and introducing potential selection biases. Inclusion criteria varied. Some studies did not exclude individuals with prior diagnosis of cancer, thus introducing a possible reverse causation bias. Other studies included both invasive and non-invasive cancers in their analyses (Bodmer *et al.* 2010). Many studies included patients exposed to a variety of treatments for diabetes complicating the analysis of metformin associations. Self-reporting of key variables such as concomitant medication use and cancer risk factors such as obesity, tobacco use and family history may have introduced exposure biases; imbalances in these factors, as well as others such as age and severity of diabetes, may have led to confounding of associations of metformin with cancer incidence or mortality. Nonetheless, it remains biologically plausible that metformin use may be causally associated with lower cancer incidence and mortality. However, caution should be exercised in translating these observations to non-diabetic patients because their physiological milieu and the cancers they develop may differ in important ways from the situation in diabetic patients. Notably, insulin resistance (characterised by high levels of circulating insulin and, in later stages, by high glucose levels with or without sustained high insulin levels) may have been present for many years before the clinical diagnosis of diabetes (Kendall & Bergenstal 2001), leading to the development of insulin- and/or glucose-dependent cancers that are more sensitive to insulin- and/or glucose-lowering effects of metformin. Because of this, it is possible that similar physiological and/or anti-cancer effects may not be present in non-diabetics.

Clinical

Retrospective clinical data suggest that metformin use in diabetics may be associated with the development of cancers that differ from those seen in the absence of metformin. Specifically, in breast cancer, metformin use has been associated with tumours that are more

Table 1 Epidemiological studies examining the association of metformin use by diabetics with cancer incidence and mortality

Study type	Outcome	Sample size	Comparison	Site	Results (HR/OR/RR and 95% CI)	Reference
Case-control	Incidence	11 876	Any vs no metformin	Multiple	OR 0.86 (0.73–1.02)	Evans <i>et al.</i> (2005)
Retrospective cohort	Mortality	10 309	Metformin	Multiple	HR 1.0	Bowker <i>et al.</i> (2006)
			Insulin		HR 1.9 (1.5–2.4)	
			Sulphonylureas (SU)		HR 1.3 (1.1–1.6)	
Case-control	Incidence	390	Any vs no metformin	Multiple	OR 0.28 (0.13–0.57)	Monami <i>et al.</i> (2009)
Retrospective cohort	Incidence	8170	Any vs no metformin	Bowel	HR 0.60 (0.38–0.94)	Libby <i>et al.</i> (2009)
	mortality					
Retrospective cohort	Incidence	62 809	SU	Lung	HR 0.70 (0.43–1.15)	Currie <i>et al.</i> (2009)
				Breast	HR 0.60 (0.32–1.10)	
				Overall	HR 0.63 (0.49–0.81)	
				Breast	HR (relative to metformin users)	
					B 0.98 (0.69–1.41)	
					C 1.80 (1.29–2.53)	
					P 4.95 (2.74–8.96)	
					R 1.07 (0.76–1.49)	
			Metformin + SU		B 0.90 (0.67–1.21)	
					C 1.43 (1.05–1.94)	
					P 0.38 (0.13–1.12)	
					R 1.18 (0.89–1.57)	
			Insulin-based		B 1.07 (0.79–1.44)	
					C 1.69 (1.23–2.33)	
					P 4.63 (2.64–8.10)	
					R 1.10 (0.79–1.52)	
Prospective cohort	Mortality	1353	Any vs no metformin	Multiple	HR 1.47 (1.22–1.76)	Landman <i>et al.</i> (2010)
Case-control	Incidence	973	Insulin	Pancreas	OR 5.04 (2.38–10.7)	Li <i>et al.</i> (2009)
			Insulin secretagogues		OR 1.74 (0.80–3.77)	
			Thiazolidinediones		OR 1.65 (0.71–3.87)	
			Metformin		OR 0.41 (0.19–0.87)	
Case-control	Incidence	1001	Any vs no metformin	Prostate	OR 0.56 (0.32–1.00)	Wright & Stanford (2009)
Case-control	Incidence	1573	Insulin or SU	Liver	OR 2.99 (1.34–6.65)	Donadon <i>et al.</i> (2009)
			Metformin		OR 0.33 (0.1–0.7)	
Case-control	Incidence	1524	Biguanides	Liver	OR 0.3 (0.2–0.6)	Hassan <i>et al.</i> (2010)
			Thiazolidinediones		OR 0.3 (0.1–0.7)	
			SU		OR 7.1 (2.9–16.9)	
			Insulin		OR 1.9 (0.8–4.6)	
Case-control	Incidence	305	Any vs no metformin	Breast	OR 0.44 (0.24–0.82)	Bodmer <i>et al.</i> (2010)
Prospective cohort	Incidence	480 984	Any vs no metformin	Colorectal	HR 0.36 (0.13–0.98)	Lee <i>et al.</i> (2011)
				Liver	HR 0.06 (0.02–0.16)	
				Pancreas	HR 0.15 (0.03–0.79)	
				Overall	HR 0.12 (0.08–0.19)	
Retrospective cohort	Mortality	595	Any vs no metformin	Colorectal	HR 1.45 (1.08–1.93)	Lee <i>et al.</i> (2012)
Retrospective cohort	Mortality	3685	Metformin	Multiple	HR 0.56 (0.34–0.94)	Bo <i>et al.</i> (2012)
			Insulin		HR 1.56 (1.22–1.99)	
Case-control	Incidence	10 781	Metformin	Ovarian	OR 0.61 (0.30–1.25)	Bodmer <i>et al.</i> (2011)
			SU		OR 1.26 (0.65–2.44)	
			Insulin		OR 2.29 (1.13–4.65)	
Nested case-control	Incidence	8098	Any vs no metformin	Prostate	RR 1.23 (0.99–1.52)	Azoulay <i>et al.</i> (2011)
Case-control	Incidence	4323	Any vs no metformin	Breast	OR 0.81 (0.63–0.96)	Bosco <i>et al.</i> (2011)
Case-control	Incidence	1340	Metformin	Multiple	OR 0.46 (0.25–0.85)	Monami <i>et al.</i> (2011)
			SU		OR 0.75 (0.39–1.45)	

B, Breast Cancer; C, Colorectal Cancer; P, Pancreatic Cancer; R, Prostate Cancer; HR, Hazard Ratio; RR, Risk Ratio; OR, Odds Ratio.

frequently progesterone receptor (PgR) positive (Berstein *et al.* 2010a) and less frequently triple negative (Meiers *et al.* 2010), whereas in lung cancer, metformin use has been associated with a higher frequency of adenocarcinomas (vs other histologies; Mazzone *et al.* 2010, Tan *et al.* 2011).

Similar retrospective data suggest that cancer outcomes may be better in diabetics with breast or lung

cancer if they are receiving metformin. Jiralerspong *et al.* (2009) studied pathological complete response (pCR) rates in breast cancer patients receiving neoadjuvant chemotherapy – pCR was significantly higher in diabetics receiving metformin than in diabetics not receiving metformin (24 vs 8%), with intermediate pCR in non-diabetics who were not

exposed to metformin (16%). Although imbalances in covariates such as age, obesity, insulin and taxane use were present, differences in pCR were significant in adjusted analyses; however, metformin use was not significantly associated with survival. Likewise, metformin use by patients with triple-negative breast cancer was not associated with beneficial effects on distant metastasis-free survival, recurrence-free survival or overall survival (Bayraktar *et al.* 2012). However, patients receiving metformin had a slightly lower risk of distant metastases (compared with diabetics not receiving metformin and non-diabetics). In a retrospective review of lung cancers in diabetics, Mazzone *et al.* (2010) reported more frequent occurrence of metastatic disease at diagnosis in those receiving insulin or thiazolidinediones (which also lower insulin; 20 vs 42% in those not receiving these agents, $P=0.027$) and a reduced risk of death in those receiving metformin (hazard ratio (HR) 0.56, $P=0.056$). Recent evidence also suggests that chemotherapy outcomes may be improved by metformin in diabetic non-small cell lung cancer patients (Tan *et al.* 2011). Because of the retrospective design of these studies and the non-random allocation of metformin (and thiazolidinediones), these results should be considered as hypothesis generating. Nonetheless, they suggest that clinically important beneficial effects may result from metformin use in cancer patients.

Prospective data regarding metformin use in non-diabetic cancer patients are beginning to emerge. We have reported a 22% reduction in insulin levels when metformin is given to non-diabetic breast cancer survivors in a standard clinical dose of 1500 mg/day (Goodwin *et al.* 2008). Hadad *et al.* (2011) have recently completed a preoperative, randomised, 'window of opportunity' trial examining the effects of metformin on non-diabetic women with operable invasive breast cancer. Metformin (1000 mg twice daily) was well tolerated and caused a significant reduction in Ki-67 staining in tumours and altered the expression of numerous genes including those involved in metabolism, inflammation and AMPK (PRKAA) and mTOR signalling. Interim data from additional ongoing neoadjuvant studies on breast cancer have provided evidence that metformin administration (in standard clinical dose) to newly diagnosed breast cancer patients is safe and has favourable effects on tumour cell proliferation and apoptosis in the absence of other anti-cancer treatment (Bonanni *et al.* 2010, Niraula *et al.* 2010). Furthermore, Hosono *et al.* (2010) have reported a randomised trial showing that a very low dose of metformin (250 mg daily) reduces proliferation and aberrant crypt foci formation in the rectal epithelium of non-diabetic patients with previous colorectal polyps when compared with placebo. Completion of these studies, as well as planned and

ongoing studies on the metastatic and adjuvant setting in breast, prostate, pancreatic and endometrial cancer (clinicaltrials.gov), will provide important additional information on the anti-cancer effects of metformin and help elucidate the clinically important mechanisms of metformin action in diabetic and non-diabetic cancer patients.

Metabolic

Obesity has been associated with increased overall cancer risk and increased risk of most common solid tumours and haematological malignancies (Calle *et al.* 2003); lung cancer is a notable exception to this association. Although many factors have been postulated to mediate effects of obesity on cancer, including sex hormones such as oestrogen, adipocytokines such as leptin and adiponectin, and inflammatory cytokines such as interleukins and tumour necrosis factor α (TNF α), recent research has focused on insulin (or insulin resistance as part of the metabolic/insulin resistance syndrome) as a potentially important mediator. In a number of meta-analyses (Everhart & Wright 1995, Larsson *et al.* 2005, 2006, 2007, Mitri *et al.* 2008, Larsson & Wolk 2011), type 2 diabetes (associated with a period of hyperinsulinaemia) has been associated with increased risk of common solid tumours including those of the breast, colon/rectum, pancreas and kidney but not the prostate (Kasper & Giovannucci 2006, Kasper *et al.* 2009). Furthermore, higher insulin or C-peptide (cleaved from proinsulin when insulin is released) levels have also been associated with risk of breast, colorectal and other cancers (Pisani 2008). These observations provide credence to an insulin-lowering mechanism of metformin action in cancer prevention.

In the setting of established cancer, higher levels of insulin or C-peptide have been independently associated with poor outcomes (increased risk of recurrence and death) in breast and prostate cancers (Goodwin *et al.* 2002, Pisanisi *et al.* 2006, Ma *et al.* 2008, Emaus *et al.* 2010, Duggan *et al.* 2011, Erickson *et al.* 2011, Irwin *et al.* 2011, Pritchard *et al.* 2011). Our group has demonstrated a more than doubled risk of distant recurrence and tripled risk of death in women with locoregional breast cancer whose insulin levels were in the highest quartile, even though those levels were within the normal range (Goodwin *et al.* 2002). In prostate cancer, this association may be limited to men with body mass index (BMI) $>25 \text{ kg/m}^2$ (Ma *et al.* 2008).

The presence of insulin receptors (IRs) on many cancers provides a biological rationale for the effects of insulin on cancer risk and prognosis – recent understanding of the nature of these receptors and the signalling pathways they activate when ligand binding occurs is reviewed in the following section.

Preclinical evidence

Mechanism of action of metformin as an anti-cancer agent

The role of metformin in the inhibition of cancer is postulated to be associated with both direct and indirect effects of the drug, as shown in Fig. 2. The indirect effects are linked to the ability of metformin to inhibit the transcription of key gluconeogenesis genes in the liver and stimulate glucose uptake in muscle, thus increasing insulin sensitivity and reducing blood glucose and lowering insulin levels. The direct effects of metformin are believed to be primarily mediated by activation of AMPK, a serine/threonine protein kinase involved in regulating cellular energy metabolism, leading to a reduction in mTOR signalling and protein synthesis in cancer cells. Metformin activates AMPK by inhibiting complex I of the mitochondrial respiratory chain, which leads to impaired mitochondrial function and conditions that effectively mimic cellular energy stress (El-Mir *et al.* 2000, Brunmair *et al.* 2004). Examples of these stresses include glucose deprivation, hypoxia, oxidative stress, ischaemia and muscle contraction or exercise, all of which lead to an increase in the ratio of AMP:ATP and activation of

AMPK (Kahn *et al.* 2005). The resulting increase in AMP activates AMPK by at least three separate mechanisms (Long & Zierath 2006): i) allosteric activation, ii) promotion of activation-specific phosphorylation of the α catalytic subunit on residue Thr172 by the upstream kinase LKB1 (STK11) and iii) interference with dephosphorylation of AMPK Thr172 by protein phosphatases. Upon activation, AMPK phosphorylates a number of effectors leading to the activation of ATP-generating pathways, such as glycolysis and fatty acid oxidation, and the inhibition of ATP-consuming pathways, such as fatty acid and protein synthesis (Kahn *et al.* 2005).

Direct (insulin-independent) effects of metformin

Protein synthesis is one of the most energy-consuming processes in the cell and, as such, is a major target of AMPK signalling under conditions of cellular energy stress. Upon activation by metformin, AMPK phosphorylates TSC2, stimulating its GTPase-activating protein (GAP) activity towards the small GTPase RHEB, which causes a reduction in mTOR signalling (Inoki *et al.* 2003). mTOR controls protein synthesis by regulating the phosphorylation of key proteins involved in mRNA translation, such as the 4E-BPs, S6Ks and the initiation factor eIF4G. Inhibition of mTOR signalling leads to a reduction in phosphorylation of its major downstream effectors, the 4E-BPs and S6Ks, and a net inhibition of global protein synthesis (Zakikhani *et al.* 2006, Dowling *et al.* 2007; Fig. 2). Reduced protein synthesis is thought to be an important mechanism of direct metformin action in the inhibition of cancer cell proliferation. Indeed, the growth-suppressive effect of metformin in breast cancer cell lines was associated with a suppression of mTOR signalling and a general inhibition of protein synthesis (Zakikhani *et al.* 2006, Dowling *et al.* 2007).

Considering the complexity of the molecular cascade mediating the effect of metformin on protein synthesis, the status of several factors can impact metformin sensitivity. For instance, in mouse embryonic fibroblasts (MEFs), loss of TSC2 leads to resistance to the effects of metformin on mTOR signalling, protein synthesis and cell proliferation (Dowling *et al.* 2007). Nevertheless, a recent report demonstrates that in MEFs, AMPK can directly suppress mTOR in the absence of TSC2 by phosphorylating the mTOR-associated adaptor protein raptor (Gwinn *et al.* 2008). The tumour suppressor LKB1 is also an important determinant of metformin sensitivity. As indicated earlier, LKB1 is the immediate AMPK activator in response to cellular energy stress and phosphorylates a key AMPK residue (Thr172) obligate for catalytic activity. Cells lacking LKB1 exhibit decreased AMPK activation and elevated mTOR signalling even under conditions of cellular energy stress or treatment with AMPK agonists (Shaw *et al.* 2004). LKB1

Figure 2 Mechanism of action of metformin in cancer. The anti-cancer activity of metformin is associated with direct and indirect effects of the drug. The direct insulin-independent effects of metformin are mediated by activation of AMPK and a reduction in mTOR signalling and protein synthesis in cancer cells. The tumour suppressors LKB1 and TSC2 are important in mediating the effects of metformin on AMPK and mTOR respectively; however, metformin may also inhibit mTOR independently of LKB1, AMPK and TSC2. The indirect insulin-dependent effects of metformin are mediated by its ability to activate AMPK and inhibit gluconeogenesis in the liver and stimulate glucose uptake in muscle. The resulting reduction in circulating insulin alleviates activation of PI3K/AKT/mTOR signalling in cancer cells. The cell membrane transporter OCT1 is required for metformin uptake in tissue and therefore may play a key role in efficacy.

is also required for metformin-mediated AMPK activation. A variety of cells lacking LKB1, including those derived from breast and cervical cancers and mouse embryonic stem cells, are resistant to the growth inhibitory effects of metformin *in vitro* (Zakikhani *et al.* 2006, Dowling *et al.* 2007, Huang *et al.* 2008). Moreover, LKB1 is required for liver AMPK activation and the associated reduction in blood glucose in response to metformin treatment (Shaw *et al.* 2005). Thus, LKB1 and TSC2 represent key factors in determining tumour sensitivity to the effects of metformin.

Increased mTOR-dependent protein synthesis and cell growth are hallmark features of tumorigenesis downstream of the activated PI3K/AKT signalling pathway, one of the most frequently deregulated molecular networks in human breast cancer. Activating mutations in the PI3K catalytic subunit (PIK3CA) have been found in 20–35% of primary breast specimens (Bachman *et al.* 2004, Lee *et al.* 2005), while mutations or loss of the tumour suppressor PTEN, a negative regulator of the PI3K/AKT cascade, has been found in up to 40% of breast tumours (Sansal & Sellers 2004, Perez-Tenorio *et al.* 2007). In addition, over-expression of HER2 (ERBB2), an upstream activator of PI3K, is observed in 30% of human breast cancers (Yu & Hung 2000). Notably, PI3K/AKT signalling correlates with breast cancer progression and contributes to resistance of breast cancer cells to chemotherapy, trastuzumab and tamoxifen (Zhou *et al.* 2004, Morgensztern & McLeod 2005). A number of physiological stimuli, including a variety of growth factors and hormones such as insulin, also activate PI3K signalling in cancer cells. Considering the prevalence of genetic changes leading to elevated mTOR signalling, targeting this cascade using metformin represents a possibly viable therapeutic option in breast cancer.

In addition to mTOR, other targets of AMPK may also be implicated in cancer cell proliferation and survival. For example, AMPK phosphorylates p53 (a tumour suppressor that is a downstream target of AMPK) upon energy stress in MEFs leading to activation of a cell cycle checkpoint, allowing cells to survive periods of energy deprivation (Jones *et al.* 2005). p53 may also impact sensitivity to metformin. Metformin inhibited the growth of p53-deficient human colon carcinoma cells *in vitro* and in tumour xenografts in mice but had no effect on p53 wild-type cells (Buzzai *et al.* 2007). This difference in sensitivity was attributed to the ability of p53 wild-type cells to undergo a metabolic adaptation, which maintained cell survival during periods of metformin-mediated AMPK activation. However, p53 status did not impact metformin sensitivity in other cancer cell lines (Zhuang & Miskimins 2008, Alimova *et al.* 2009). Sensitivity to the anti-tumoral effects of metformin may also be governed by expression levels of the organic cation transporters (*OCT1* (*SLC22A1*), -2

(*SLC22A2*), and -3 (*SLC22A3*)). *OCT1* is primarily responsible for metformin transport into cells and polymorphisms in the gene encoding *OCT1* that affect hepatic uptake of metformin *in vitro* and have been associated with decreased efficacy in patients (Shu *et al.* 2007, Jin *et al.* 2009, Tzvetkov *et al.* 2009, Memmott *et al.* 2010). *OCT1* is highly expressed in liver tissue, with lower levels present in the kidney and minimal levels present in the lung and small intestine (Jin *et al.* 2009, Tzvetkov *et al.* 2009, Memmott *et al.* 2010). Little is known about the expression of *OCT1* in human tumours; however, an assessment of its expression patterns in tumour tissue will be critical to elucidate the mechanism of anti-tumour action of metformin and the identification of patients best suited for metformin therapy.

Recent reports have raised the possibility that metformin exhibits additional inhibitory effects on gluconeogenesis and mTOR signalling independent of AMPK and TSC2 (Gwinn *et al.* 2008, Foretz *et al.* 2010, Kalender *et al.* 2010). Metformin inhibited mTOR activity in cells lacking TSC2 and AMPK by suppressing RAG GTPases, which are involved in mTOR activation (Kalender *et al.* 2010). Furthermore, metformin lowered hepatic gluconeogenesis in the absence of AMPK, or its kinase, LKB1, by reducing hepatic energy levels (Foretz *et al.* 2010). These observations highlight the need for additional research focusing on the mechanism of action of metformin. Nevertheless, it appears likely that the ability of metformin to exert direct effects on mTOR signalling and indirect effects on circulating insulin remain integral to its potential mechanism of action in the treatment of cancer.

Indirect (insulin-dependent) effects of metformin

As noted earlier, metformin may also exhibit indirect effects on tumour growth by reducing circulating glucose and insulin levels via inhibition of hepatic gluconeogenesis and stimulation of glucose uptake in muscle (Fig. 2). High insulin levels associated with obesity, insulin resistance and type 2 diabetes may promote tumorigenesis via activation of the foetal isoform of the IR-A, which is highly expressed in human breast cancer. The IR lies upstream of a number of growth-promoting pathways including the PI3K/AKT/mTOR signalling network (Belfiore & Frasca 2008, Pollak 2008). The IGF1R, also proposed to be involved in breast cancer, is less highly expressed in breast tumours but may hybridise with IR-A to bind insulin and stimulate proliferation of breast cancer cells at concentrations that do not activate IGF1R dimers (Papa & Belfiore 1996, Mulligan *et al.* 2007). Together, these observations suggest that, unlike normal adult epithelial cells, breast cancer cells may acquire sensitivity to the growth and survival effects of insulin.

Reduction in insulin levels by metformin may counteract this sensitivity by reducing ligand binding to IR-A/IGF1R, indirectly inhibiting tumour growth and improving breast cancer outcomes.

***In vivo* models of anti-tumour activity of metformin**

The effects of metformin on spontaneous breast tumour development have been examined in MMTV-*Her2/Neu* transgenic mice that exhibit mammary-specific expression of the HER2/NEU oncoprotein, resulting in the almost universal development of focal mammary adenocarcinomas, which metastasise to the lungs. Metformin treatment, in a dose comparable to that used clinically in humans, significantly delayed the appearance of mammary adenocarcinomas, reduced the size of tumours and prolonged the lifespan of MMTV-*Her2/Neu* mice (Anisimov *et al.* 2005). These effects were accompanied by a significant reduction in blood glucose and a statistically non-significant diminution in circulating insulin levels (Anisimov *et al.* 2005). In mice heterozygous for the tumour suppressor PTEN (highly prone to tumours in various organs), metformin (administered at a dose of 300mg/kg body weight per day, more than tenfold higher than the standard clinical dose) delayed tumour onset by 25% (Huang *et al.* 2008), an effect that required LKB1-mediated AMPK activation. Metformin was also effective in reducing the growth of intestinal polyps in mice deficient for the tumour suppressor APC (Tomimoto *et al.* 2008).

Orally administered metformin (at plasma levels (2.7–10.3 µM) equivalent to those achieved in patients (2.8–15 µM)) also reduced tobacco carcinogen-induced lung tumourigenesis (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)) in mice (tumour burden reduced by 53%; Memmott *et al.* 2010). Intraperitoneal administration, resulting in higher plasma levels of metformin (24.2 µM), reduced tumour burden by 72%. Although AMPK activation was observed in the liver and circulating insulin levels were reduced after i.p. injection, metformin failed to activate AMPK in lung tumour tissue. A concomitant reduction in phosphorylation of IGF1R/IR, AKT and ribosomal protein S6 in tumours suggests that the indirect (insulin-mediated) effects of metformin were responsible for the reduction in lung tumour burden.

In addition to spontaneous and carcinogen-induced tumours, metformin has been found to impact growth in mouse xenograft and syngeneic tumour models – the tumour cells injected in these models were grown in culture media that contained high levels of glucose, insulin and other growth factors. In at least two syngeneic model systems (Lewis lung carcinoma LLC1 cells in C57BL/6J mice and triple negative 66c14 cells in Balb/c mice), metformin (at doses comparable to human doses in the former and ~40 times higher than

human doses in the latter) reduced tumour growth when mice were fed a high-energy diet (which induces weight gain and insulin resistance; Algire *et al.* 2008, Phoenix *et al.* 2010). In these models, the high-energy diet enhanced tumourigenesis, whereas metformin significantly reduced the effect of this diet on tumour growth. Metformin improved insulin sensitivity in tumour-bearing high-energy diet fed mice and resulted in AMPK activation and reduced IR-mediated signalling in tumours. Paradoxically, metformin failed to inhibit the growth of tumours in mice on a control diet, despite activation of AMPK in tumour tissue (Algire *et al.* 2008). These observations are consistent with an indirect, insulin-lowering mechanism of metformin action; however, it must be recognised that the tumour cells were dependent on high insulin levels in culture medium before their injection into mice and mechanisms may differ in the clinical setting in which this dependence is not present.

***In vitro* models of anti-cancer effects of metformin**

In vitro work focusing on the effects of metformin on breast cancer cells demonstrated that metformin inhibited the growth of a variety of breast cancer cells regardless of oestrogen receptor (ER), PR, HER2 or p53 status (Zhuang & Miskimins 2008, Alimova *et al.* 2009). Recently, it was reported that metformin induced unique responses in the triple-negative (ER, PR and HER2 negative) breast cancer cell line MDAMB-231, leading to an S phase cell cycle arrest, reduced cell proliferation and colony formation, as well as increased apoptosis (Liu *et al.* 2009). In this system, metformin also induced AMPK activation, reduced the phosphorylation of epidermal growth factor receptor (EGFR), MAPK and Src and lowered the levels of cyclins D1 and E (Liu *et al.* 2009). Growth inhibitory effects were recapitulated *in vivo* where metformin significantly reduced the growth of triple-negative breast cancer cell xenografts in nude mice. Some of the anti-cancer effects of metformin on triple-negative breast cancer cells were also associated with STAT3 inhibition. Treatment of triple-negative cells with metformin led to a reduction in STAT3 activation and downstream signalling and metformin acted synergistically with a STAT3 inhibitor to decrease cell growth and induce apoptosis (Deng *et al.* 2012). Metformin may also be effective against ER-positive breast cancers by inhibiting aromatase expression in tumour stroma. In primary human breast adipose stromal cells, metformin treatment led to an increase in the phosphorylation of AMPK, which was associated with an inhibition of nuclear translocation of CRTC2, a CREB co-activator known to increase aromatase expression (Brown *et al.* 2010). Furthermore, metformin interacted additively with tamoxifen to reduce

breast cancer cell proliferation (Berstein *et al.* 2010b). These results indicate that metformin may represent a potentially effective therapy in women with ER-positive breast tumours.

Emerging evidence indicates that breast cancer cells over-expressing *Her2*, either through gene amplification or ectopic expression, exhibit increased sensitivity to the growth inhibitory effects of metformin (Vazquez-Martin *et al.* 2009). The effects of metformin were associated with translational suppression of HER2 protein expression mediated by the inhibition of S6K1, a downstream effector of mTOR (Vazquez-Martin *et al.* 2009). Of interest, the mTOR inhibitor rapamycin caused similar translational suppression of erbB3 (an EGFR, also known as HER3 in humans) in breast cancer cells and mammary tumours in mice (Liu *et al.* 2005). Taken together, these data indicate that metformin and other inhibitors of mRNA translation act by reducing translation of mRNAs encoding specific growth factor receptors. Metformin also exhibited inhibitory effects on trastuzumab-resistant breast cancer cells. In trastuzumab-resistant models, metformin disrupted ERBB2/IGF1R complexes and significantly reduced cell proliferation (Liu *et al.* 2011).

Metformin has also been reported to exert anti-proliferative effects against a number of other cancer cell types including those derived from prostate, endometrial and brain tumours (Isakovic *et al.* 2007, Ben Sahra *et al.* 2008, Cantrell *et al.* 2010). In prostate cancer cells, metformin treatment caused a G1 cell cycle arrest (mediated by a reduction in cyclin D1 levels and phosphorylation of Rb), as well as an increase in the levels of the cell cycle inhibitor p27kip, resulting in a strong anti-proliferative effect both *in vitro* and in tumour xenografts in mice (Ben Sahra *et al.* 2008). In endometrial cancer cells, metformin induced a G1 arrest and at high doses caused apoptosis while suppressing mTOR signalling (Cantrell *et al.* 2010). Finally, while causing cell cycle arrest in low-density cultures of rat glioma cells, metformin induced apoptosis in confluent cell cultures (Isakovic *et al.* 2007).

A major obstacle in the effective treatment of breast and other cancers is the resistance of tumour cells to drug therapy. The existence of tumour-initiating (stem) cells, a specific subset of tumour cells that are believed to be involved in tumour initiation, progression, heterogeneity and recurrence, has been proposed as one of the underlying causes of this resistance (Campbell & Polyak 2007, Polyak & Weinberg 2009). Metformin specifically inhibited the growth of CD44⁺/CD24^{lo} (a putative stem cell marker signature; Hirsch *et al.* 2009) cells in culture and reduced their ability to form tumours when injected into nude mice. When metformin was combined with doxorubicin, a striking reduction in both CD44⁺/CD24^{lo} and other cancer cells was observed; this combination was also

effective in preventing tumour growth and relapse in mice (Hirsch *et al.* 2009, Iliopoulos *et al.* 2011). Significantly, no CD44⁺/CD24^{lo} cells could be isolated from mice after metformin treatment, raising the possibility that metformin may specifically target tumour-initiating cells. Metformin was also effective in preventing tumour relapse in mice when combined with the standard chemotherapeutic agents paclitaxel and carboplatin, highlighting the potential application of metformin as part of a combinatorial therapeutic strategy (Iliopoulos *et al.* 2011). However, in some experiments the regimen of metformin administration complicates the interpretation of results (Hirsch *et al.* 2009). Large volumes (up to 0.5 ml) of metformin solution were directly injected into tumour xenografts, likely resulting in exposure of tumour cells to non-physiological doses of the drug, as well as necrosis (due to direct tumoral injection). It is unclear how a comparable drug delivery can be achieved in patients. Nonetheless, these data imply a possible benefit in combining metformin with standard chemotherapies in breast cancer treatment.

Limitations of preclinical models

One of the major limitations in interpreting many preclinical studies is the high concentration of metformin used *in vitro* in relation to the concentration that can be safely obtained in the clinical setting (Fig. 3). Most *in vitro* studies report using doses of metformin between 1 and 40 mM (165–6600 mg/l), which is well above the feasible therapeutic plasma levels (0.465–2.5 mg/l or 2.8–15 µM) in humans (Stambolic *et al.* 2009). Thus, it is possible that metformin caused a degree of energy stress (and resulting AMPK activation) in these studies that far exceeds effects that would be seen clinically. For example, Phoenix *et al.* (2009); reported pro-angiogenic effects of

Figure 3 Concentrations of metformin used in clinical and preclinical studies. The anti-cancer effects of metformin have been tested over a wide range of doses. Clinical and epidemiological studies have utilised metformin at standard doses of up to 2250 mg/day. Conversely, preclinical studies often involve extremely high, non-physiological concentrations of metformin that are in excess of the therapeutic levels achieved in human patients.

metformin in a mouse xenograft model; however, metformin concentrations were hundreds of times higher than those that are deemed safe clinically; it is possible that these extremely high concentrations led to intracellular metabolic stresses (and responses to those stresses) beyond those that would be seen clinically, raising questions as to whether such pro-angiogenic effects are clinically relevant.

Nonetheless, recent studies indicate that metformin is effective at reducing cancer cell proliferation *in vitro* at concentrations as low as 10 μ M, well within the therapeutic range of the drug (Liu *et al.* 2009). Moreover, metformin is capable of inhibiting tumour growth in mouse models at doses equivalent to those used in humans without causing toxicity (Anisimov *et al.* 2005, Ben Sahra *et al.* 2008). Importantly, only slight levels of AMPK activation are required to trigger downstream signalling events (Hawley *et al.* 2002).

Another concern arises from the growth conditions of the cell culture models used to assess the inhibitory effects of metformin *in vitro*. The majority of cancer cell lines are grown in culture media that contains extremely high amounts of growth factors and glucose. For example, average tissue culture media contains glucose at concentrations between 10 and 25 mM, well above the fasting levels of glucose observed in non-diabetic patients (<6 mM). Most media is also supplemented with 5–10% foetal bovine serum, which contains high concentrations of growth factors and hormones, including insulin and EGF. Thus, cancer cell lines are often maintained in non-physiological conditions that are optimised for maximum growth and proliferation. The excessive concentrations of insulin, glucose and growth factors in culture media, combined with the presence of oncogenic mutations and variable expression of the membrane transporter OCT1, may account for the elevated doses of metformin required to elicit cellular responses *in vitro*.

A host response is not present *in vitro*, and as a result, the direct (insulin-independent) mechanism(s) of metformin action have been the focus of this research. Because many of the conditions evaluated in this *in vitro* work are outside the boundaries possible in the clinical setting, it is conceivable that many of the direct effects of metformin reported in *in vitro* work will not be relevant in the clinical situation.

Thus, although a beneficial anti-cancer effect of metformin at lower concentrations that are encountered clinically remains plausible, mechanistic effects identified in the preclinical studies may differ from those that are most relevant in the clinical setting. It is therefore necessary to exercise caution in further studies of the effects of metformin *in vitro*. Careful dosing, equivalent to therapeutic levels *in vivo*, and strategies for varying the levels of insulin and

glucose within culture media are required for physiological modelling of metformin *in vitro*. It is anticipated that such models will be more likely to inform clinical studies.

Translational challenges

The anti-cancer effects of metformin are associated with both direct (insulin-independent) and indirect (insulin-dependent) actions of the drug. This duality of action has increased the complexity of clinical evaluation of metformin, including the translation of preclinical observations. Existing preclinical, clinical and epidemiological evidence has incorporated a broad range of insulin, glucose and metformin concentrations and has often focused on different potential mechanisms of metformin action (Fig. 3). Effects that have been identified in preclinical work, including differential effects in different molecular subtypes of breast cancer, although plausible, require confirmation in the clinical setting. Focused research in the neoadjuvant and metastatic settings, with biopsies pre- and post-metformin administration, and concurrent evaluation of physiological effects will help to elucidate the clinical relevance of these effects.

Differentiation of the relative importance of indirect (insulin-mediated) and direct (insulin-independent) metformin effects is critical for optimal evaluation of this agent. The postulated mechanisms of action give rise to a broad range of therapeutic targets, leading to a large number of potential clinical markers of metformin benefit (Table 2). Understanding the relative importance of therapeutic targets and markers of benefit is required for appropriate selection of patients who might benefit from metformin. For example, if indirect insulin-mediated effects of metformin predominate, patient selection should target individuals with

Table 2 Predictors of metformin benefit in human cancer

Marker	Indirect effect (insulin-dependent)	Direct effect (insulin-independent)
Host		
High BMI	+	
Physical inactivity	+	
High fasting insulin	+	
Insulin resistance	+	
OCT1/2/3 expression (liver)	+	
Tumour		
IR/IGF1R expression	+	
Increased PI3K/mTOR	+	+
OCT1/2/3 expression		+
LKB1 expression		+
TSC2 expression		+

high insulin levels and/or obesity, germline expression of OCTs (particularly *OCT1*) and tumour characteristics including IR/IGF1R expression and PI3K pathway activation. In contrast, if direct (insulin-independent) mechanisms of metformin action are key, tumour characteristics such as LKB1 and TSC2 expression, OCT1 positivity and PI3K/AKT/mTOR activation will likely emerge. It is possible that the predominant mechanism of metformin action (and key markers of activity) will differ across types of tumours. Once key mechanisms of action (and their associated markers) are understood, metformin can be used in a targeted fashion, incorporating patient and tumour attributes consistent with known mechanisms of action.

Although evidence of direct (insulin-independent) anti-cancer effects of metformin that has arisen from preclinical research is strong, leading to a focus on these direct effects by many researchers, emerging clinical and *in vivo* data suggest that indirect insulin-mediated effects of metformin may be of key importance in at least some common cancers, including breast and lung. If this is borne out by ongoing and future research, the focus of preclinical research will need to shift to models that incorporate these indirect effects, requiring more complex designs than are currently used in most *in vitro* research. Similarly, investigation of markers of metformin benefit will need to include key host factors such as circulating insulin and glucose levels, obesity and germline OCT1 expression, in addition to attributes of tumour cells such as insulin/IGF1 receptor expression that can potentially mediate these indirect, host-mediated effects and any direct effects that are relevant (Table 2).

Going forward, care is required to maximise the efficiency of evaluation of metformin as a potential anti-cancer agent. The known safety profile of the agent allows rapid evaluation and has led to the initiation of at least one large-scale Phase III adjuvant trial (NCIC CTG MA.32) in the breast cancer setting (Goodwin *et al.* 2011). Several neoadjuvant studies are ongoing, and studies on the metastatic and prevention settings are ongoing and planned in a number of cancer types. It is essential that these studies include strong embedded correlative research components, with evaluation of host and tumour factors to identify potential predictors of metformin benefit and, more importantly, to allow enhanced understanding of the relative contributions of indirect insulin-mediated and direct insulin-independent metformin action. The absence of pharmaceutical industry interest in metformin (due to its generic status) has led to less co-ordination of research activities than is commonly seen in anti-cancer drug development – as a result, it is essential that the research community ensures that this function is fulfilled and that a major focus on clinical translation and relevance emerges in future research.

Declaration of interest

P J G is the principal investigator and V S is the correlative science chair of a Phase III adjuvant metformin trial (NCIC CTG MA.32). Apotex Canada, a generic drug company, supplies drug and placebo to NCIC CTG for use in this trial.

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