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A Review

A review on repurposing anti-diabetic drugs for the amelioration of betel-nut induced carcinogenesis

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Betel-nut (BN) chewing is a socially ingrained practice in several populations worldwide, and is the fourth most addictive habit with several detrimental impacts on health. BN is classified as a class I carcinogen, and its use is associated with the development and progression of cancer through various mechanisms such as the production of nitrosamines, increased oxidative stress, activation of the PI3K/Akt/mTOR pathway, inhibition of tumor suppressors and the dysregulation of cellular energetics, apoptosis and autophagy. Several of these mechanisms overlap with the pathogenesis of diabetes mellitus, making anti-diabetic drugs good candidates for drug repurposing as anti-cancer agents. While a large body of experimental evidence has established the effectiveness of the anti-diabetic drug, metformin, as an anti-cancer agent, clinical trials have largely failed to produce promising results. Our own work as well as that of other workers have also indicated the anti-cancer potential of the dipeptidyl peptidase-4 inhibitor, vildagliptin, though the thiazolidinedione, pioglitazone, was found to have more limited effectiveness and also produced ambiguous results in clinical studies. This review focuses on the mechanisms of BN-induced carcinogenesis and the potential of exploring common anti-diabetic drugs for its amelioration.

Keywords: Anti-diabetic drugs, Betel-nut, Cancer, Drug repurposing

Introduction

Areca nut, popularly known as betel-nut (BN), is the kernel of the tropical palm, *Areca catechu*[1,2]. The earliest remains of *Areca catechu* dating from 10,000 BC have been found in Northwestern Thailand, and archaeological and philological evidences indicate that this palm species may have originated in Malaysia, while the use of BN as a masticatory is likely to have emerged in the Indonesian archipelagos[3]. BN paired with the leaf of the vine, *Piper betle,* referred to as 'tambula', entered India via the South Sea islands-Java and Sumatra, during the early Gupta period[3]. The use of BN in Southeast Asian countries dates back to the 11th century when it was symbolically used to solidify relationships in the royal kingdoms. Over the years, it has found diverse roles among different Southeast Asian communities in religious ceremonies and customary practices[4]. In the present day, extensive use of BN for chewing purposes is prevalent among the populations of India, Bangladesh, Pakistan, Nepal, China, Taiwan, Bhutan, Cambodia, Maldives, Malaysia, Indonesia and Philippines[5]. In the past few decades, BN use has also grown rapidly in the Western countries owing to the increased migration of Asians to these countries[6].

In India, BN is most frequently consumed in the form of betel-quid (BQ), also known as *paan*, comprising a mixture of BN, tobacco, and slaked lime wrapped in betel leaf. It is rampantly used by both males and females across various age groups in various forms, including widely marketed products such as *paan masala*, *supari mixture* and *gutka*[7]. *Paan* users claim to have a sense of well-being, heightened alertness, and increased stamina and it is also used as a mouth freshener in several Indian communities[1,7].

Habitual chewing of BN is considered to be one of the four most addictive habits worldwide[8]. In India, approximately 8.3% of men and 7.7 % of women consume BN in various forms[9]. The habit is accompanied by various detrimental effects on oral and overall health, and is significantly associated with the development of oral potentially malignant disorders (OPMDs) such as oral leukoplakia (OL), erythroplakia, or oral submucous fibrosis (OSF), oral cancer, and cancers of the oesophagus, liver, pancreas, larynx and lungs. In fact, the International Agency for Research on Cancer (IARC) has classified BQ with or without tobacco as a Group I carcinogen[10]. Despite the health

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implications, BN use is a socially acceptable, enormously deep-rooted habit in several communities, prevalent among both males and females of all age groups, regardless of socio-economic classes[1,2]. Although BN has a long history in traditional medicine, no clinical trials have been conducted to validate this point[11].

Drug repurposing or repositioning is an innovative approach adapted by researchers globally as a means for substantially reducing the time and cost required to develop a new drug. Repurposing already approved drugs promises a shortened development cycle along with a significant reduction in production cost[12]. Traditional drug discovery requires a novel molecule to undergo stringent investigations due to the unavailability of clinical and toxicological data[13]. On the other hand, a repurposed molecule can skip the pre-clinical stages and directly enter phase II of the drug development process[12]. Therefore, with prior knowledge regarding safety, efficacy, and route of administration, a repositioned drug can be successfully brought to the market by reducing the development cost and time. The anti-diabetic drug metformin is being widely explored for its potential application as an anticancer agent attributed to its functions in multiple signaling pathways, including activation of AMP-activated kinase (AMPK)[14-16]. The anti-diabetic drugs vildagliptin and sitagliptin are reported to exhibit anti-cancer properties when used in a colon cancer cell line[17], while pioglitazone and rosiglitazone have shown anti-cancer effects in breast cancer[18,19], human leukemia[20] and pancreatic[21] cancer cell lines, and in cohort studies on lung[22] and breast cancer[23].

This review aims to provide an overview of the mechanisms of betel-nut induced carcinogenesis and the potential of repurposing anti-diabetic drugs for its amelioration based on experimental evidence from our laboratory.

Mechanisms of betel-nut induced carcinogenesis

BN induces carcinogenesis through the complex interaction of its various chemical constituents with cellular components, to promote the development of the cancer phenotype. Some of the mechanisms of BN-induced carcinogenesis are listed below.

(a) BN alkaloids form carcinogenic nitrosamines

Evidence from chemical analysis indicates that betel-nut has significant alkaloid content, comprising arecoline (2.22 mg/g dry weight), guvacine (2.48 mg/g dry weight), guvacoline (0.99 mg/g dry weight) and arecaidine (0.15 mg/g dry weight)[24]. BN chewing results in the formation of specific nitrosamines in the oral cavity by the nitrosation of arecoline producing areca-nut/betel-nut specific nitrosamines such as N-nitrosoguvacoline (NGL), N-nitrosoguvacine (NGC) 3-N-nitrosomethylaminopropionitrile (NMPN), 3-N-nitrosomethylaminopropionaldehyde (NMPA) and 3-methylnitrosaminopropionitrile (MNPN). Of these, NGL, NGC and MNPN[25-27] have been detected in the saliva of BN-chewers. In an *in vivo* study, subcutaneous administration of NMPA caused lung adenocarcinoma, nephroblastoma, and leukaemia in rats[28] indicating the association of this nitrosamine with carcinogenesis. These N-nitrosamines undergo enzymatic α-hydroxylation by cytochrome P-450 (CYP450) monooxygenases resulting in the formation of diazonium ions[29]. Rearrangement and subsequent elimination of nitrogen result in the formation of carbenium ions, the final DNA alkylating species which cause highly mutagenic and cytotoxic *O*-alkylations producing O^4-methylthymine (O^4-MeT), O^6-methylguanine (O^6-MeG) and O^6-ethylguanine (O^6-EtG)[30]. Human CYP450 was found to be involved in the mutagenic activation of NMPN, NMPA and NG using genetically engineered *Salmonella typhimurium* YG7108 expressing each form of human P450 together with NADPH-P450 reductase. CYP2A6 and CYP2A13 are the two members of the human P450 enzyme group responsible for the mutagenic activation of NMPN and NMPA, respectively. The expression of CYP2A13 increases in the respiratory tracts of BN chewers and is reported to activate pro-mutagen NMPA to its genotoxic form[31].

(b) BN increases oxidative stress

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and their elimination by antioxidants, resulting in accumulation of ROS. It is responsible for a wide array of ROS-induced molecular damage with pathological consequences, including the development of cancer[32]. The different chemical constituents of BQ contribute to oxidative stress through different mechanisms. The ROS-induced lesion, 8-hydroxy-2'- deoxyguanosine (8-OH-dG), was reported to be produced upon exposure of DNA to BQ ingredients and ripe BN extract under alkaline conditions, *in vitro*[33,34]. Polyphenols present in BN extract produce superoxide ions that lead to the formation of 8-OH-dG[35]. The BN alkaloid, arecoline, disrupts cellular redox balance by activating NADPH

oxidase (NOX), cyclooxygenase-2 (COX-2), sirtuin 3 (SIRT3) and endothelial nitric oxide synthase (eNOS), and inhibiting glutathione (GSH), catalase and superoxide dismutase (SOD)[36]. Long-term exposure to BN leads to the accumulation of genetic mutations that generate ROS[37] and depletion of GSH level in human keratinocytes[38] and the stomach of BQ exposed diabetic rats[39]. Hydroxychavicol, a phenolic component of betel leaf, induced 8-OH-dG formation and DNA single strand breaks in cultured cells, and is found in the saliva of human BQ chewers at a concentration of 4.6 mM after chewing[40]. High levels of ROS are associated with DNA fragmentation, lipid peroxidation and protein carbonylation, leading to cellular damage[41]. In our studies on a murine model of BN-induced carcinogenesis, the liver, lungs, spleen and small intestinal tissues of mice exposed to arecoline and aqueous extract of betel-nut (AEBN) had significantly elevated levels of lipid peroxidation and protein carbonylation, indicating the accumulation of oxidative stress[14-16, 36].

(c) Betel nut induces genetic instability

BN and its components are genotoxic agents which damage DNA and induce genetic instability through multiple ways. Exposure to arecoline as well as BN extract damaged the DNA of bone marrow cells of Swiss albino mice, leading to the formation of micronuclei (MN), chromosomal aberrations (CA) and sister chromatid exchange (SCE)[42,43]. The frequency of CA and SCE increased when arecoline was administered concomitantly with GSH depletion by treatment with the GSH synthesis inhibitor, buthioninesulfoximine (BSO). Arecoline arrested cells at prometaphase leading to distorted organization of mitotic spindles and misalignment of chromosomes[44]. Arecoline was also found to be mutagenic in transgenic mouse by increasing the frequency of both transition and transversion mutations at G:C sites[45]. Areca nut extract increased the frequency of MN and induced mutations in human keratinocytes[46]. Chromosomal instability characterized by increased frequency of precocious anaphase and aneuploid cells was induced in bone marrow cells of mice exposed to raw areca nut extract[47]. Ethyl acetate and *n*-butanol extracts of areca nut induced chromosomal aberrations in Chinese hamster ovary cells. Aqueous extracts of betel leaf induced chromosomal aberrations in human lymphocytes. Aqueous, *n*-Butanol and ethyl acetate extracts of betel leaf also induced chromosomal aberrations and in Chinese hamster ovary cells. Furthermore, BQ chewers from India and the Philippines had elevated frequencies of micronucleated buccal mucosal cells at the site within the oral cavity where they regularly kept the BQ[25].

Several studies have investigated the underlying factors responsible for the increased DNA damage, decreased DNA repair and overall genetic instability induced by BN/BQ, and loss of tumor suppressor response has emerged as a pivotal factor. Arecoline decreased tumor suppressor activity in human epithelial cells by inhibition of expression and transactivation functions of the tumor suppressor, p53[48]. This may be correlated to arecoline induced hypermethylation at the promoter of *p53* gene that subsequently resulted in low levels of p53 protein in oral submucous fibrosis[49]. AEBN decreased the expression of total p53[50], total Brca1 and Brca2[51] and phosphorylated p53-Ser15[52,15,36] in the tissues of mice. In oral squamous cell carcinoma (OSCC), BN consumption led to murine double minute 2 (MDM2) mediated downregulation of the p53 protein[53,54]. MDM2 is a negative regulator of p53 and is seen to be overexpressed in human tumors[53]. Decreased expression of mitotic and spindle checkpoint genes *AuroraA, AuroraB, MAD2* and *Bub1* was also reported to be a significant contributing factor for inducing chromosomal instability in mice exposed to raw areca nut extract[47]. Areca nut extract induced overxpression of miR-23a which correlated with reduced expression of the Fanconi anemia susceptibility gene, *FANCG*, that would compromise DNA double-strand break breaks (DSB) repair[55]. Chinese patients with areca nut-associated OSCC had a genomic signature characterized by DNA mismatch repair deficiency[56]. BN consumption is also linked to the methylation of the promoter of the gene coding for runt-related transcription factor 3 (RUNX3), a well known tumor suppressor protein[57].

(d) Betel-nut deregulates cellular proliferation

Uncontrolled cellular proliferation is a hallmark of cancer[58]. In an *in vitro* study, BN extract increased proliferation and induced DNA damage of mouse kidney cells[59]. BN extracts have been shown to increase the proliferation of different human cell lines at varying concentrations[60]. Arecoline increased cellular proliferation in the prostate gland of male Wistar rats, resulting in prostatic hyperplasia and hypertrophy confirmed by increased expression of the proliferation marker, Ki-67, and concomitant with overexpression of the cell cycle regulatory proteins,

Cyclin D1 and CDK4[61]. AEBN also induced Ki-67 overexpression with increased expression of Cyclin D1 in the liver of AEBN exposed mice, indicating increased cellular proliferation[15,16,36,52]. Arecoline induces cell proliferation through various mechanisms. In the human OSCC cell line, arecoline treatment increased the expression of c-Myc protein, a principal inducer of cell proliferation and suppressed the expression of miR-22, which has tumor suppressor functions[62]. In another study, arecoline was found to mediate tumorigenesis in OSCC through peroxiredoxin-2 (PRDX2)[63] which promotes tumor survival and induces cell proliferation[64].

(e) Betel nut induces loss of apoptosis

Apoptotic cell death plays a vital role in preventing tumorigenesis, restraining tumor growth and preventing drug resistance[65]. Interestingly, the effect of BN and its constituents on apoptosis is ambiguous, with both pro-apoptotic and anti-apoptotic effects reported. Arecoline has shown pro-apoptotic effects in cultured human basal cell carcinoma cells (BCC-1/KMC) by activating caspase-3 and inhibiting B-cell leukemia/lymphoma protein 2 (Bcl-2)[66], in rat cardiomyocytes by inducing Fas ligand as well as mitochondria dependent cell death[67] and in human HaCaT epithelial cells by increased expression and activation of cleaved-Bid, cleaved-PARA and cleaved-caspase-3[68].The apoptotic effect of arecoline was enhanced by co-treatment with the tea antioxidant epigallocatechin-3-gallate (EGCG) in prostate cancer cells line[69], and with melatonin in OSCC cells[70], respectively. In one study, arecoline treatment inhibited apoptosis in HepG2 cells by upregulating cyclin dependent kinase 1 (CDK1)[71].

BN extract has, however, displayed both anti-apoptotic and pro-apoptotic effects depending on cell type, treatment duration, and dosage. BN extract induced caspase dependent apoptosis in oral cancer cell lines, HSC-2 and HSC-3[72]. Areca nut extract also induced apoptosis of thymocytes *in vivo* when administered intraperitoneally, through activation of caspase-3 and Apoptosis Inducing Factor (AIF)[73]. On the other hand, areca nut extract produced anti-apoptotic effect on neutrophils attributed to the inactivation of caspase-3 and caspase-8[74]. In other studies BNE induced cell-cycle arrest but not apoptosis in oral KB epithelial cells[75], and normal human oral keratinocytes (NHOK)[76]. We previously reported that treatment of Swiss albino mice with AEBN inhibited apoptosis by upregulating the anti-apoptotic protein, Bcl-2[15,16,36,52]. It has been reported that a fraction of areca nut extract induced autophagy in different types of carcinoma cells, while arecoline triggered caspase-3 mediated apoptosis. This difference was attributed to the ability of areca nut extract, but not arecoline, to inhibit the phosphorylation of the mTOR-Ser2448[77]. This finding is consistent with that from our group, since we observed increased expression of the *mTOR* gene concomitant with significantly increased ratio of pmTOR-Ser2448/total mTOR in the tissues of AEBN treated mice when compared to the untreated control, but decreased expression of the *mTOR* gene with only a marginal increase in the ratio of pmTOR-Ser2448/total mTOR in arecoline treated mice[16,36,52].

(f) Betel nut dysregulates the PI3K/Akt/mTOR pathway

The phosphoinositide 3 kinase (PI3K)/Akt/ mammalian (or mechanistic) target of rapamycin (mTOR) pathway is a highly conserved signal transduction pathway among eukaryotes. Dysregulation of this pathway occurs in more than 50 % tumors and involves hyperactivity of PI3K, loss of PTEN, gain-of-function of p53, and activation of mTOR, which drive cancer development and progression by promoting cell survival, cell growth, and cell cycle progression[78]. The information on the role of BN in deregulating this pathway is limited. In 2007, it was reported that BN extract contributed to oral carcinogenesis via Akt-mediated downregulation of involucrin[79]. Another study revealed PI3K/Akt mediated upregulation of vimentin in OSCC cells following treatment with BN extract[80]. In head and neck squamous cell carcinoma (HNSCC) cells, vimentin expression is associated with aggressiveness[81] and it results in poor prognosis for breast cancer[82]. Moreover, in rat hepatocytes, arecoline treatment activated Akt/mTOR pathway by phosphorylating Akt at Ser-473 and mTOR at Ser-2448[83]. The tea polyphenol, EGCG, has been reported to inhibit the proliferation and colony formation of arecoline-induced esophageal squamous cell carcinoma (ESCC) cells by inhibiting AKT and ERK1/2 pathway[84]. Our studies revealed that the treatment of mice with AEBN produced carcinogenesis with nodulation of the liver, significantly upregulated phosphorylation of Akt at Ser-473 and mTOR at Ser-2448[16,36,52] and significantly downregulated PTEN[85], indicating that dysregulation of the PI3K/Akt/mTOR pathway plays an important role in BN-induced carcinogenesis.

(g) Betel nut induces autophagy

Autophagy is a tightly regulated process which serves to degrade unnecessary or dysfunctional cellular components and to recycle metabolic substrates in response to environmental and cellular stresses[86]. Autophagy plays a dual role in oncogenic development depending on the tissue type and stage of tumorigenesis. It aids in the survival and growth of cancer cells in advanced stages by regenerating essential biomolecules to counter nutrient deprivation, while in the early stages, it promotes cancer cell death by inducing tumor suppressor activity[87,86]. It is induced by the co-ordination of two nutrient sensing kinases, the AMPK-activated kinase (AMPK) and mTOR (86). AMPK directly phosphorylates autophagy-related proteins in the mTORC1, ULK1, and PIK3C3/VPS34 complexes[88] to induce autophagy, while mTOR inhibits autophagy through inhibitory phosphorylations at ULK1 and Atg 13[89]. Thus, the inhibition of mTOR by nutrient deprivation or pharmacological agents such as rapamycin can stimulate autophagy[90]. The hallmarks of autophagy are the cleavage of the precursor form of microtubule associated protein 1 light chain 3 (LC3-I) to the active form LC3-II, and the emergence of autophagic vacuoles (AV) and acidic vesicles[77]. Chronic exposure to a 30-100 KDa fraction of areca nut extract resulted in LC3-II accumulation, appearance of intrcellular vacuoles and acidic vesicles in the oral cancer cell lines OECM-1 and CE81T/VGH[91] indicating the induction of autophagy, and stimulated the expression of beclin-1 in CE81T/VGH cells, thereby contributing to tumor growth through the upregulation of autophagy[92]. Transmission electron microscopy studies of liver nodules induced in Swiss Albino mice by transgenerational exposure to AEBN revealed enhanced cristolysis of mitochondria and formation of acidic vesicles[93]. BN chewing was also associated with chemoresistance in a cohort of OSCC patients who exhibited increased expression of LC3-II and resistance to the chemotherapeutic drug, cisplatin[94].

(h) Betel-nut dysregulates the AMPK pathway and induces dyslipidemia

The AMPK signaling pathway plays a pivotal role in maintaining metabolic homeostasis through a wide array of direct targets and processes, and its dysregulation has been implicated in the development of a number of diseases, including obesity, diabetes and cancer. The master regulator of the pathway is the sensor of intracellular adenosine nucleotide levels, AMPK[95]. AMPK is activated by phosphorylation at Thr-172 and acts as a metabolic tumor suppressor by enforcing metabolic checkpoints through its action on molecules that regulate cellular growth and metabolism[96]. Chronic exposure to arecoline or BN was reported to alter the activation of AMPK in a variety of experimental systems. In 3T3-L1 preadipocyte cell, arecoline suppressed the phosphorylation of AMPK at Thr-172 in a dose dependent manner[97]. Arecoline also elevated intracellular ROS levels and inhibited AMPK phosphorylation at Thr-172 in a time- and dose-dependent manner in a number of cell types including oral (OECM-1) and esophageal (CE81T/VGH) cell lines[98]. Our *in vivo* studies found that arecoline as well as AEBN reduced AMPK activity by lowering its phosphorylation at Thr-172, and concomitantly induced carcinogenesis in arecoline (14) and AEBN treated mice[14-16,36,52].

The dephosphorylation of AMPK reprograms cellular metabolism leading to upregulated lipid synthesis which promotes oncogenic progression[99,100]. A higher prevalence of metabolic syndrome among the BN chewing female population of Karachi, Pakistan in comparison to males belonging to the same population, has also been reported[101]. *De novo* fatty-acid synthesis involves two key enzymes, acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FASN). ACC carboxylates acetyl-CoA to form malonyl-CoA, which is further converted by FASN to long-chain fatty acids[102]. Phospho AMPK (Thr-172) induces downstream inhibitory phosphorylation of ACC1 resulting in lowered production of malonyl-CoA and consequent inhibition of fatty acid synthesis[103]. Furthermore, AMPK also phosphorylates and inactivates the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGCR), resulting in inhibition of cholesterol synthesis[104]. Our findings reveal that AEBN or arecoline treatment inhibited AMPK phosphorylation leading to downstream activation of ACC and HMGCR. The expression of FASN was also upregulated in the liver of arecoline and AEBN exposed mice. These molecular alterations resulted in dyslipidemia with significant increase in levels of total serum triglycerides and cholesterol and significant decrease in levels of high density lipoproteins (HDL). Thus, BN-induced cancer may be fueled by reprogrammed lipid metabolism, which drives carcinogenesis and cancer progression[105].

The link between diabetes mellitus and cancer

The interconnecting link between diabetes mellitus and cancer has been recognized for more than eight decades, and a large body of clinical and epidemiological studies have firmly established that diabetes patients are at a high risk for liver, pancreas and endometrial cancer followed by colorectal, breast and bladder cancer[106]. Various factors have been implicated to interlink diabetes mellitus and cancer. These include altered insulin and insulin-like growth factor (IGF) signals, obesity, inflammation, hyperglycemia, metabolic syndrome, ER stress and autophagy[107]. Insulin resistance leads to hyperinsulinemia, that is, an elevated level of circulating insulin, which in turn causes a rise in the level of free and bioactive insulin-like growth factor 1 (IGF-1)[106]. It is clinically associated with a number of diseases including obesity, type 2 diabetes mellitus, metabolic syndrome, cardiovascular diseases, and cancer[108]. Insulin and insulin like growth factor 1 (IGF-1) subsequently bind to insulin receptor (IR) and IGF-1 receptor (IGF1R), respectively, activating intrinsic receptor tyrosine kinase activity of the receptors to elicit downstream signaling cascades through the activation of the PI3K-Akt pathway and the Shc-Ras-MAPK pathways. Activation of these pathways regulates gene transcription, glucose, lipid and protein metabolism, cell growth and differentiation, initiation and maintenance of cancer stem cells and promotes protection from apoptotic stimuli[109] thereby promoting cancer development. Overexpression of insulin receptors has been reported in various types of cancers such as breast, thyroid, and prostate cancer cells[110,111]. Increased blood glucose level (hyperglycemia) associated with diabetes mellitus has been reported to contribute towards cancer progression through various mechanisms. Hyperglycemia increases the production of free radicals and other reactive molecules that cause oxidative damage of DNA, which is an initial step in oncogenesis. It also results in inhibition of the antioxidant function of thioredoxin through action of the glucose-inducible gene, thioredoxin-interacting protein (TXNIP). Hyperglycemia causes high rates of protein glycation and the formation of advanced glycation end products (AGEs) which contribute directly to oxidative stress by damaging proteins and the extracellular matrix. Furthermore, AGE bind to the receptor for advanced glycation end products (RAGE), leading to the activation of various downstream processes including thrombogenesis, angiogenesis, and vascular inflammation via Ras-extracellular signal regulated kinase-nuclear factor-kappa pathways which increase oxidative stress and chronic inflammation, favoring the progression of both cancer and diabetes[112]. Other mechanisms through which hyperglycemia contributes towards cancer development and progression are increasing the levels of insulin/ IGF-1 and inflammatory cytokines in the blood, increasing leptin and Akt/mTOR signaling, enhancement of Wnt/ β-catenin signaling, and promoting epithelial mesenchymal transition (EMT)[112]. Chronic inflammation in poorly managed diabetes mellitus stimulates high levels of inflammatory markers including tumor necrosis factor- α (TNF-α) and interleukin-6 (IL-6), which enhances the risk of cancer incidences[113,114]. Obesity is an established risk factor for both type 2 diabetes mellitus and cancer. Obesity is associated with an increased level of various pro-inflammatory molecules which promote cancer development and progression. Leptin has mitogenic, pro-inflammatory, anti-apoptotic and pro-angiogenic activity[112], and high levels in obese individuals increase the risk for breast[115] and colon cancer[116]. High levels of interleukin-6 (IL-6) in obese individuals have also been associated with hepatocellular carcinoma by activating PI3K/Akt and Janus kinase/signal transducers and activators of transcription-3 (JAK/STAT3) pathways[117,118]. Resistin is a pro-inflammatory cytokine secreted from macrophages. It mediates insulin resistance and was initially discovered as a link between diabetes and obesity. Elevated resistin levels have been reported in obesity-influenced cancers such as breast and colon cancers, as well as in cancers irrespective of obesity and has been linked to increased risk of progression, angiogenesis, metastasis, chemoresistance and stemness induction in cancer[119]. Other pro-inflammatory molecules involved in oncogenesis which are reported at high levels in obese individuals are tumor necrosis factor-α (TNF-α), retinol binding protein-4, plasminogen activator inhibitor-1 (PAI-1) and hepatic growth factor (HGF)[112]. Conversely, there is a decreased secretion of adipokines such as adiponectin and visfatin in adipose tissues. Adiponectin boosts insulin sensitivity, and is essential for the AMPK mediated inhibition of tumor formation[120]. Thus, low circulating levels of adinopectin are inversely related to cancer development and progression[121] and several obesity related cancers such as endometrial, colorectal, and

postmenopausal breast cancers[122] have low levels of adiponectin.

Anticancer potential of anti-diabetic drugs

The past few decades have seen extensive research on the anticancer potential of various classes of anti-diabetic drugs[14,123] based on the rationale that diabetes and cancer share several interlinking mechanisms which may be targeted by the same drugs. Some of the results obtained from repurposing these drugs in *in vitro* and *in vivo* studies, and in clinical trials have been greatly encouraging. However, various clinical trials have also yielded ambiguous results.

Metformin

Metformin (1,1-dimethylbiguanide) is a biguanide anti-diabetic drug, and the most frequently prescribed anti-diabetic medication[14]. It is non-toxic, reduces insulin resistance, lowers plasma fasting insulin level, and is also effective in the treatment of obesity, hyperlipidemia and some cardiovascular complications. Furthermore, it has been shown to lower the risk for thyroid cancer, gastric cancer, breast cancer, oral cancer, prostate cancer, colorectal cancer and ovarian cancer in diabetics taking the drug (reviewed in 112). Metformin exerts anti-cancer effects directly by activating AMPK. It inhibits the mitochondrial respiratory chain complex I to restrict ATP production and create a condition similar to cellular energy stress which leads to activation of AMPK. Activated AMPK, in turn, inhibits the mammalian target of rapamycin complex 1 (mTORC1) pathway. The inhibition of mTOR signaling has a great potential for anti-cancer therapy as mTORC1 is upregulated in the majority of cancers[112]. Metformin can also inhibit tumor cell proliferation by disrupting p706SK mediated insulin/IGF-1 signaling pathway[121]. Furthermore, metformin exerted anti-cancer effects by upregulating miR-497 which acts as a tumor suppressor in esophageal cancer[124], and miR-192-5p and miR584-3p which inhibited cell growth and suppressed cell motility in melanoma[125]. Metformin inhibits tumor progression through various immunomodulatory effects, including suppressing the nuclear factor kappa β (NF-κβ)[126] and IL-6 signaling[127] pathways, promoting the generation of cluster of differentiation-8 (CD8) cells[128] and strengthening T-cell immunity[129]. Metformin induced apoptosis in pancreatic cancer cells by reducing the expression of p300/CBP-associated factor (PCAF)[130]. A recent review paper[131] discussed several clinical trials, where metformin effectively reduced the risk of colorectal, gastric, esophageal and pancreatic cancers. However, metformin failed to produce promising results in clinical trials designed to evaluate its effect on overall survival (OS) of prostate cancer patients in four phase II clinical trials[132-135] raising doubts about the clinical benefits of metformin in cancer patients.

Pioglitazone

Pioglitazone, is an anti-diabetic drug of the glitazone or thiazolidinedione class and functions to improve insulin sensitivity of the peripheral tissues. It is an agonist of the nuclear hormone receptor known as peroxisome proliferator-activated receptor gamma (PPARγ), and exerts anti-cancer effects through PPARγ dependent as well as independent mechanisms[112,136]. The PPARγ dependent antiproliferative effect of pioglitazone was reported in human renal cell carcinoma where activation of PPARγ by pioglitazone decreased the expression of cyclin D1 leading to cell arrest, decreased the expression of antiapoptotic protein Bcl2 and increased the expression of Bax protein to induce apoptosis[137]. Pioglitazone also activated the PD-1/PD-L1 immune checkpoint and induced PD-L1 autophagic degradation in a PPARγ-dependent manner[138]. Pioglitazone significantly reduced the expression of matrix metalloproteinase 2 (MMP-2) protein in human adrenocortical cell line, H295R, thereby restricting invasion of tumor cell[139]. Also, pioglitazone exhibited its anti-carcinogenic effect by inhibiting the expression of mitogen activated protein kinase (MAPK) and NF-κB in a rat model for prostate cancer[140]. Moreover, pioglitazone, when administered in combination with doxorubicin, enhanced the cytotoxic effect of the chemotherapeutic drug by significantly reducing the migration of MDA-MB-231 cells[141]. Pioglitazone treatment effectively regressed the diethylnitrosamine (DEN)-induced liver fibrosis in male Wistar rats by activating AMPK signaling pathway[142]. In thyroid cancer cells, C643 and SW1736, pioglitazone exhibited anticancer potential via AMPK mediated inhibition of mTOR protein, which eventually stimulated apoptosis[143]. Thus, while experimental evidence indicates the anti-cancer potential of pioglitazone, clinical trials have thrown up more ambiguous results. Although several clinical trials reported alleviating effects of pioglitazone on different cancer types, others have shown that long term pioglitazone use increases the risk for bladder

cancer[136]. Hence, cohorts including global population with their response to different doses and duration of pioglitazone administration are needed before pioglitazone can be recommended for clinical use.

Vildagliptin

Vildagliptin is an anti-diabetic drug belonging to class of dipeptidyl peptidase-4 (DPP-4) inhibitors. It acts by inhibiting the DPP-4 enzyme, preventing the degradation of the incretin hormones, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), which subsequently stimulate the pancreatic α and β cells and regulate insulin secretion depending upon blood glucose concentration[144]. Although DPP-4 inhibitors have been in clinical use with significant advantages and negligible side effects, there have been concerns regarding their association with pancreatitis and pancreatic cancer, albeit without much evidence[145]. Unlike other anti-diabetic drugs, the anticancer potential of vildagliptin has been investigated only recently resulting in limited information available. Vildagliptin exhibited its anticancer potential *in vitro* in colon cancer H-29 cells[17], and in high fat diet (HFD)-induced liver cancer by preventing angiogenesis[146]. Vildagliptin also prevented the mobilization of leukemic stem cells in diabetic mice, suggesting its efficiency in restricting metastasis[147]. In an *in vivo* study, vildagliptin treatment stimulated innate immune response in colorectal and lung metastatic mouse model by promoting macrophage and NK-cell mediated shrinkage of tumors formed by Lewis Lung Carcinoma (LLC) cells and human lung adenocarcinoma (H460) cells injected subcutaneously into C57BL/6 and CD1/nude mice[148]. It also stimulated C-X-C motif chemokine ligand 10 (CXCL10) mediated production of pro-inflammatory cytokines that led to the enhanced cytotoxicity of natural killer (NK) cells and T-cells in a model of hepatocellular carcinoma[149].

Anti-cancer effects of anti-diabetic drugs in a murine model of betel-nut induced carcinogenesis

Our studies revealed that treatment of female Swiss albino mice with the BN alkaloid, arecoline, or the BN extract, AEBN for 16 weeks resulted in formation of preneoplastic nodules on the liver and lesions on the lungs, which were exacerbated after 24 weeks of treatment. Significant tissue dysplasia was observed with disruptions in tissue architecture of the liver, lungs, small intestine, stomach and tongue. Biochemically, the arecoline and AEBN exposed mice exhibited dyslipidemia and significant increase in levels of oxidative stress. Treatment of arecoline/AEBN exposed mice with the anti-diabetic drugs metformin, vildagliptin or pioglitazone for 8 weeks resulted in a dose-dependent decline of liver nodulation and restoration of tissue architecture. Lipid peroxidation and protein carbonylation were lowered, and normolipidemia was restored in a dose-dependent manner. Apoptosis was also restored with a significant increase in the ratio of apoptotic to non-apoptotic cells. Ki-67 immunoreactivity of liver tissue declined significantly upon treatment with metformin and vildagliptin, but not pioglitazone[14-16,36,52,85].

Mechanistically, these ameliorative effects were accompanied by the activation of AMPKα through increased phosphorylation at Thr-172 induced by all three drugs. This was concomitant with decreased phospho Akt (Ser 473) and phospho mTOR (Ser 2448) levels, pointing towards a mechanism wherein AEBN treatment promoted oncogenic signaling by activation of the Akt/mTOR pathway, which was diminished upon treatment with metformin, vildagliptin and pioglitazone. Indeed, it has been reported that the treatment of normal human oral keratinocyte (NHOK) with areca nut extract (ANE) resulted in activation of Akt[79]. Activated Akt encourages cell growth and survival by phosphorylating its downstream protein complex, mTOR complex 1 (mTORC1), at Ser-2448. Phosphorylated mTORC1 regulates the activation of p70S6K and inactivates 4EBP1, which in turn promotes anabolic activities such as lipid synthesis, glucose metabolism, and protein synthesis[150] essential for tumor growth and cancer metastasis. Previous workers have reported that AMPK plays a vital role in regulating Akt in response to stress, and it is proposed that activation of AMPK suppresses the phosphatidylinositol 3-kinase (PI3K)/Akt pathway[151,152]. Loss of PTEN is a significant event in activation of the PI3K/Akt pathway[153]. Thus, activation of AMPK and restoration of PTEN protein expression by anti-diabetic drugs may play a pivotal role in reducing Akt/mTOR signaling to ameliorate BN-induced carcinogenesis[14-16,36,52,85].

Activated AMPK phosphorylates p53 on Ser15, leading to its sustained activation, which may, in turn lead to cellular senescence or apoptosis[154]. In our studies, phosphorylation of p53 at Ser-15 was restored effectively by metformin and vildagliptin[14-16,36,52], but activation of AMPK by pioglitazone failed to

upregulate the levels of phosphorylated p53 (Ser-15)[52,85]. Overexpression of the anti-apoptotic protein Bcl-2 prevents p53-induced apoptosis suggesting that Bcl-2 can regulate p53-dependent cell death[155]. Akt-mediated phosphorylation and subsequent inactivation of the pro-apoptotic protein, BAD, encourages the optimal functioning of the pro-survival protein Bcl2[156], to promote cell death resistance[157]. Pioglitazone-induced inactivation of Akt may have thus induced apoptosis in our model by decreasing Bcl2 expression, while metformin and vildgaliptin induced apoptosis by restoring the levels of p53 (Ser-15).

Cyclin D1 induces cell cycle progression by forming a complex with cyclin dependent kinase 4 (Cdk4)[158]. Phosphorylation of p53 at Ser-15 induces the downstream expression of p21, which in turn degrades cyclin D1 and discourages the formation of cyclin d1/Cdk4 complex[159,160]. In our studies, decrease in Ki-67 expression in liver tissue upon treatment with metformin and vildagliptin was concomitant with significant decline in levels of Cyclin D1, indicating that metformin and vildagliptin induced p53-mediated decline in the expression of Cyclin D1 protein to restrict cell proliferation[15,16,36,52]. However, treatment with pioglitazone failed to bring any noticeable change in cell proliferation, attributable to the unavailability of phosphorylated p53 (Ser-15)[52,85].

Finally, metformin, vildagliptin and pioglitazone effectively restored normal lipid metabolism in our model through AMPK activation, concomitant with increased downstream inhibitory phosphorylation of ACC at Ser-79, and significantly reduced levels of FASN and HMGCR, thereby restoring normolipidemia and ultimately reducing BN-induced carcinogenesis. These findings are consistent with those of other workers in different models. AMPK activation by metformin has been reported to improve lipid metabolism in an animal model of chronic atrial fibrillation animal model[161]. Metformin also induced a decline in FASN levels and lipogenesis in prostate cancer cells[162]. A meta-analysis study suggested that DPP-4 inhibitors elicited lipid-lowering action by reducing cholesterol levels significantly[163]. The DPP-4 inhibitor, sitagliptin, reduced plasma lipoprotein levels and body weight in type 2 diabetic patients[164,165] and regulated the expression of lipid metabolism enzymes in NAFLD mice[166], and rabbits fed with an atherogenic diet[167]. Rosiglitazone and pioglitazone have been reported to reduce HMGCR mRNA levels and activate AMPK in the liver of mice indicating their alleviating effects on plasma lipid levels[168]. Pioglitazone also decreased triglyceride levels and increased the level of HDL-C in type 2 diabetic patients[169].

Conclusion

Chewing BN in different forms is a socially accepted habit widely prevalent among the populations of South-East Asia and the Pacific Islands, despite having a causal association with various diseases, including cancer. BN-induced cancer can develop in different tissues through multifarious mechanisms, though the primarily affected sites are the oral cavity and the head and neck region. It has severe social and economic implications on the affected populations of developing countries, attributable to the burgeoning prices of anti-cancer therapeutics. A repurposed drug exhibits clinical benefits for the treatment of a disease while having been previously approved for the treatment of a different set of indications. Drug repurposing effectively reduces the time and cost incurred in drug development because the pharmacokinetics, toxicology and safety data of an approved drug are already available. Anti-diabetic drugs are a class of widely prescribed drugs with established safety profiles, and experimental evidence as well as data from clinical trials supports the repurposing of some of them as anti-cancer drugs. Our laboratory established the *in vivo* anti-cancer effects of two popular anti-diabetic drugs, metformin and vildagliptin, in BN-induced cancer in a murine model. A third drug, pioglitazone, was also investigated for its ameliorative effects in our model, but the results were inconclusive due to its inability to restore p53-mediated tumor suppression and to control cellular proliferation. Promising evidence from epidemiological and experimental studies sparked a great interest in investigating the potential of repurposing metformin for anti-cancer treatment. However, a number of large randomised trials have failed to demonstrate the clinical benefits of metformin as a cancer treatment[170]. These, and the ambiguity of results obtained from pre-clinical studies and clinical trials exploring the anti-cancer potential of pioglitazone indicate that clinical trials investigating the repurposing of anti-diabetic drugs in cancer therapy will benefit from being designed with a fresh outlook. More investigations are warranted into the role of these repurposed drugs in regulating metabolism in cancer patients, their interactions with

the gut microbiota and the tumor microenvironment, and their plausible role in cancer prevention[170].

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Conflict of interest

All authors declare no conflicts of interest.

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