HUSSIEIN IRHAMA
hirhama@yahoo.com

Education and Certification:

- **Bachelor of Arts in Chemistry, (June, 1996)**
  - University Of the Pacific, Stockton, CA, USA

- **Doctor of Dental Surgery (DDS), (June, 1999)**
  - University Of the Pacific School of Dentistry, San Francisco, CA, USA

- **Master of Science degree in Biomedical Science (to be conferred July, 2015)**
  - University of Maryland School of Dentistry, Baltimore, Maryland, USA

Postgraduate Training:

- **Residency: (July 2011-2014)**
  - Advanced Education in General Dentistry (AEGD)
    - University of Maryland School of Dentistry, Baltimore, MD, USA

  - General practice residency
    - Ministry Of Public Health, Kuwait

Work Experience:

- **1999 - 2002** Valley Dental Corporation, California, USA
- **2003 - 2004** Specialties dental clinics- Kuwait Ministry of Health
- **2004 - 2005** General dental clinic– Kuwait Ministry Of Health
- **2005 - 2008** Kuwait Medical Center – Private Dental Center
- **2008-2010** General Dental Practice - Kuwait Ministry Of Health
- **Current-** Teaching experience in AGD clinic
Examinations and License:

• American national boards (I+II)
• California Dental board
• Kuwaiti Dental License
• American Board Of General Dentistry (I+II)

Professional memberships:

• American Academy of General Dentistry
• American Board of General Dentistry
• Kuwait Dental Association
**Background:** Compelling preclinical evidence indicates that the biguanide metformin, the most widely prescribed oral antidiabetic drug in the United States, prevents the progression of oral premalignant lesions into oral squamous cell carcinoma (OSCC). Metformin triggers antitumoral responses in part by inhibiting the oncogenic mammalian target of rapamycin (mTOR) pathway through activation of one of its key negative regulators and critical sensor of cellular bioenergetics, the AMP-activated protein kinase (AMPK). As a small hydrophilic cationic compound, metformin enters cells through cell membrane organic cation transporters (OCTs) belonging to the *SLC22A* gene family. Emerging evidence also shows that OCT expression is significantly reduced or absent as OSCC tumors become more aggressive and less differentiated. Therefore, we hypothesized that phenformin, another biguanide compound with a more hydrophobic structure and possibly more potent antineoplastic activity, might exert its antitumoral effects through an OCT-independent manner. This hypothesis was tested through the following specific aims: (1) To determine whether phenformin induces OSCC growth inhibitory actions; (2) To determine whether OCT function is necessary for phenformin-induced activation of the AMPK signaling pathway.

**Methods and Materials:** Cell viability assays and immunoblotting techniques were conducted by using human-derived OSCC cell lines HN6 and HN13.
**Results:** Our results demonstrated that: (1) phenformin significantly reduced OSCC cell viability, and (2) phenformin appeared to activate AMPK signaling through an OCT-independent manner.

**Conclusion:** This in vitro study shows that phenformin, in contrast to metformin, may exert a more potent antineoplastic effect since it decreased OSCC cell viability with much lower doses. In addition, phenformin appears not to be dependent on OCT expression and activity to activate AMPK signaling. Collectively, these studies suggest that phenformin might become an alternative biguanide, alone or in combination with conventional chemotherapy agents, when treating OCT-deficient OSCC tumors.
Phenformin and Oral Cancer: The Role of Organic Cation Transporters

By
Hussein Irhama

Thesis submitted to the faculty of the Graduate School of the University of Maryland, Baltimore in partial fulfillment of the requirements for the degree of Master of Science 2015
Acknowledgement

I would like to express my deepest gratitude to my mentor, Dr. Abraham Schneider for giving me the opportunity to experience a totally new and exciting field of science to me and for his ever-present help, support and guidance.

I would like to thank everyone in the seventh floor lab for their kindness, friendliness and help: Tao, Noor, David, and Therwah.

I would like to thank my lab buddies Amr and Faisal for being there and lending a hand when it was needed.

I would like to thank Dr. Douglas Barnes and Dr. Gary Kaplowitz for their support and guidance throughout my AEGD residency.

I would like to thank my sponsor: Kuwait Government and specially the cultural division at Kuwait Embassy for their help and support.

Special thanks to my wife Dr. Asma Buhamrah for always being there to motivate, encourage and push me to fulfill my goals.
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>iv</td>
</tr>
<tr>
<td>Table of contents</td>
<td>v</td>
</tr>
<tr>
<td>List of figures</td>
<td>vi</td>
</tr>
<tr>
<td>Chapter (1): Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter (2): Materials and Methods</td>
<td>8</td>
</tr>
<tr>
<td>Chapter (3): Results</td>
<td>11</td>
</tr>
<tr>
<td>Chapter (4): Discussion</td>
<td>15</td>
</tr>
<tr>
<td>Conclusion</td>
<td>22</td>
</tr>
<tr>
<td>References</td>
<td>23</td>
</tr>
</tbody>
</table>
List of Figures

Fig 1: Overall working model…………………………………………………………….7
Fig 2: Viability assays (HN6 AND HN13)………………………………………………12
Fig 3: AMPK activation in quinidine-pretreated HN6 (Phen vs. Met)…………………14
Fig 4: AMPK activation in OCT3 knocked down HN13 (Phen vs. Met)………………14
Fig 5: Galega Officinalis (French lilac)……………………………………………………15
Fig 6: Chemical structures of metformin and phenformin…………………………..17
Chapter 1

Introduction

Oral cancer: An increasingly prevalent malignancy in search of improved treatment

More than 500,000 cases of head and neck cancer, largely affecting the oral cavity and pharynx are diagnosed yearly worldwide. A total of 1,658,370 new cancer cases and 589,430 cancer deaths are projected to occur in the United States in 2015[1]. An estimated 45,780 new cases of cancer of the oral cavity and pharynx have been projected for 2015, mostly diagnosed as oral squamous cell carcinomas (OSCC) [1]. Of these new expected cases, an estimated 8,650 deaths is projected for the same year [1].

Late diagnosis and field cancerization with multifocal premalignant dysplastic lesions or secondary primary OSCCs still remain as major critical challenges in head and neck clinical oncology. These events largely compromise standard surgical treatment and chemotherapy. Thus, early cancer detection and diagnosis is crucial for improving survival rate and treatment success.

The constant identification of highly prevalent molecular signatures underlying OSCC pathogenesis is providing the basis to target the disease through mechanism-based treatments. Compelling evidence points to the mammalian target of rapamycin (mTOR) signaling network as one of the most frequently dysregulated pathways driving oral carcinogenesis [2-5]. The mTOR pathway is responsible for integrating mitogenic, nutrient- and energy-sensing cues to control cellular processes commonly involved in
human cancer such as cell growth, proliferation, differentiation, survival and angiogenesis [6]. To this end, recent information provides strong evidence on the widespread activation status of the mTOR pathway in hundreds of human OSCC specimens [7]. A number of studies have addressed the potential clinical benefit of mTOR inhibitors, such as rapamycin and its analogs, in the treatment of head and neck oral cancer [3,8-12]. For instance, the use of the mTOR inhibitor rapamycin represents a suitable therapeutic option in the management of patients with fully established or recurrent OSCC tumors. However, long-term use of this immunosuppressive drug may result in potential undesirable side effects. Long-term interventions with well-tolerated, low cost drugs may offer a much needed strategy designed to prevent the progression of a cancer type where locoregional invasion, nodal metastasis and chemo resistance are hallmarks of a devastating advanced disease [13].

**Antineoplastic actions of antidiabetic biguanide metformin**

In the past decade, a considerable number of population, preclinical and clinical studies strongly indicated that the glucose lowering drug metformin reduces cancer incidence and/or improves cancer prognosis in diabetic, and possibly in non-diabetic patients. In 2005, Evans et al. case-controlled study on 923 cases of cancer in 11,876 newly diagnosed type 2 diabetic patients revealed that cancer incidence in diabetic patients taking metformin appeared generally lower than in patients taking other antidiabetic drugs [14]. Interestingly, this study also revealed that the longer the period of treatment with metformin, the lower the cancer incidence. Diabetics taking metformin were 23%
less likely to develop cancer and this fraction rose to 40% for patients taking the drug for longer periods. Although more rigorous population studies are needed to confirm these promising outcomes, these findings stimulated many studies in different types of cancer to further explore the underlying mechanisms of action by which metformin may impact tumor progression.

**Potential mechanisms of action underlying the antineoplastic effects of metformin:**

**Role of the AMPK pathway**

Biguanides appear to trigger antineoplastic responses by inhibiting the oncogenic mammalian target of rapamycin (mTOR) pathway through the activation of the adenosine monophosphate–activated protein kinase (AMPK) signaling pathway. The AMPK pathway is a key negative regulator of mTOR signaling and an important sensor of cellular energy status [15-17]. AMPK acts as a cellular energy checkpoint that, if activated through metabolic stresses, maintains energy equilibrium by switching on alternative catabolic pathways while inhibiting energy consuming anabolic pathways, including cell growth and proliferation [18]. As a result, synthesis of proteins, cholesterol and fatty acids is turned off and catabolic processes aimed at generating energy like glucose uptake and glycolysis, as well as fatty acid oxidation, are turned on [19,20]. Metformin, and possibly phenformin, another member of the biguanide family, selectively affect tumor cell proliferation by perturbing mTOR signaling translational control of a specific subset of mRNAs encoding cell cycle regulators in an AMPK signaling-dependent manner [15,17,21]. As a mild inhibitor of mitochondrial complex I,
metformin induces AMPK activation following the inhibition of oxidative phosphorylation, which leads to an increase in cellular AMP levels [22]. AMP binds to AMPK, making AMPK a better substrate for phosphorylation by the upstream tumor suppressor serine/threonine kinase LKB1 [23]. Activated AMPK phosphorylates and activates the tumor suppressor protein TSC2, which then negatively regulates mTOR activity [24]. These findings have strongly linked AMPK activity to the negative regulation of downstream pathways relevant to the control of tumor cell proliferation and survival [25,26].

**Phenformin: another biguanide compound with potential use in oncology**

The promising effects of metformin in cancer have also sparked interest on the potential oncologic use of phenformin, another member of the biguanide family. In the 1970s, phenformin was used as an antidiabetic drug, but the Food and Drug Administration (FDA) recommended its withdrawal from the U.S.A market due to the occurrence of lactic acidosis–related morbidity and mortality in elderly patients with a history of cardiovascular disease and renal failure. Although metformin is associated with a much lower incidence of lactic acidosis in diabetics than phenformin, recent tissue culture and preclinical animal studies show that phenformin might be superior to metformin as an antineoplastic agent. This may be associated with differences in potency and pharmacokinetics [16,25,27,28]. Overall, this suggests that under proper monitoring of cancer patients, phenformin could be repurposed as an antineoplastic agent with plausibly safer outcomes when compared to the standard currently used chemotherapeutic drugs.
Organic cation transporters role in uptake of biguanides

Organic cation transporters, OCTs, are a group of polyspecific membrane transporters belonging to the SLC22A gene family [29,30]. OCT1 (SLC22A1), OCT2 (SLC22A2) and OCT3 (SLC22A3) mediate transport of structurally diverse, small hydrophilic organic cationic endogenous compounds, toxins and drugs, including metformin [31-35]. OCT1 and OCT2 expression is highly restricted to liver and kidney, respectively. In the liver, OCT1 mediates the therapeutic antidiabetic action of metformin as demonstrated by Shu et al (2007), who showed that metformin glucose-lowering effect in OCT1-deficient mice is completely abolished [35]. Genetic variations in OCT2 also alter metformin uptake kinetics and elimination [36,37]. Therefore, patients with different OCT1 or OCT2 genotypes and/or expression levels may respond differently to the therapeutic effects of metformin. OCT3 tissue distribution is much broader and a potential determinant of the peripheral effects of metformin on skeletal muscle [31].

It has been established that the biguanide metformin requires uptake by OCT into cells (figure 6) causing the subsequent activation of AMPK and are potent activators of AMPK signaling cascade [38-40]. This uptake is noted in tissues that are found to have high level of OCT 3 expression like oral dysplasia and well to moderately differentiated tumors. Where OCT3 expression was very low or lacking, AMPK activation was not observed suggesting no uptake of metformin into cancer cells [41].

Little is known about the role of OCTs on phenformin uptake and pharmacokinetics; specifically, no studies exist on phenformin’s use in oral carcinogenesis. Based on the reported findings demonstrating a progressive OCT3 loss of expression as OSCC tumors
become more atypical and less differentiated [41], phenformin, not metformin may be the potential biguanide of choice when targeting OCT deficient OSCC tumors.

**Central hypothesis**

Biguanides with more hydrophobic structures like phenformin affect OSCC cell viability and activates AMPK signaling in an OCT independent manner (Figure 1).

The present *in vitro* study tested the central hypothesis through the following specific aims:

*Specific Aim 1*

To determine whether phenformin induces OSCC growth inhibitory actions.

*Specific Aim 2*

To determine whether OCT function is necessary for phenformin-induced activation of the AMPK signaling pathway.
Figure 1. Overall working model: In contrast to metformin, a hydrophilic biguanide, the effects of phenformin on OSCC viability and AMPK signaling activation are not dependent on OCT function due to its structure which is more hydrophobic.
Chapter (2)

Materials and methods

Cell Culture and reagents

Human derived oral squamous cell carcinoma (OSCC) cell lines HN6 and HN13 were cultured in DMEM containing 4.5 g/L glucose and supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma, St. Louis, MO, USA) at 37º in humidified air with 5% CO₂. Noteworthy, prior to treatment with biguanides, medium was changed to a lower glucose concentration (1 g/L) in addition to the same supplements as described above. Metformin (1,1-dimethylbiguanide hydrochloride), phenformin (phenylbiguanide) and quinidine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

Following indicated experiments, medium was aspirated and OSCC cell monolayers were rinsed with 1X ice-cold Dulbecco’s phosphate buffered saline (D-PBS) and rapidly lysed with 1X SDS protein lysis buffer (62.6 mM Tris-HCL (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT). Lysates were sonicated and protein concentration was quantified in a spectrophotometer using the Bradford reagent (Sigma). Following protein quantification, each sample was brought up to similar volumes with deionized water and equal amounts of sample loading buffer. Samples were heated at 90-100º C for 5 Minutes. Equal amounts of protein (35-45 µg) were separated by SDS-PAGE using a 10-well pre-casted
4-20% gradient gel (mini-Protean TGX, BiorRad, Hercules, CA, USA), and electrophoretically transferred onto polyvinylidene difluoride membranes (BioRad).

Transfer efficacy and equivalent sample loading was confirmed by staining membranes with Ponceau S red solution (Sigma). Membranes were blocked in blocking buffer (5% non-fat dry milk in 0.05% Tween 20 –TBS) for 1 hour, and then replaced by the primary antibody diluted in blocking buffer for 2 hours at room temperature or overnight at 4° C, depending on the specific antibody. When indicated the following antibodies were used from Epitomics (Burlingame, CA, USA): rabbit monoclonal against OCT3 (SLC22A3) (1:10000); from Cell signaling Technology (Danvers, MA, USA): rabbit polyclonal against phosphor-acetyl-CoA carboxylase (pACC) (Ser79) (1:1000) and rabbit polyclonal against ACC (1:1000); and from Sigma rabbit polyclonal against GAPDH (1:10000); rabbit polyclonal against OCT1 (SLC22A1) (1:400), and rabbit polyclonal against OCT2 (SLC22A2) (1:250). Secondary horseradish peroxidase linked donkey anti-rabbit IgG antibodies were obtained from Amersham Biosciences (Piscataway, NJ, USA).

**Cell Viability assays**

The effect of biguanides on OSCC cell viability was evaluated through a colorimetric assay by using the CellTiter 96® Aqueous One Solution (MTS) reagent following the manufacturer’s protocol (Promega, Madison, WI). OSCC were plated overnight in triplicate in 96-well plates at a density of 10000 cells/cm². The following day, cells were either left untreated or exposed to metformin or phenformin. After 72 hours, MTS reagent was added directly to each culture well, incubated at 37° C for 1-4 hours and absorbance at 490 nm was measured in a microplate reader.
**RNA interference**

Short interfering RNA (siRNA)-based experiments were performed as previously published [42]. Briefly, HN13 cells were plated at 15,000 to 20,000 cells/cm\(^2\) overnight in complete DMEM. The following day, transfections were performed following the dilution of commercially available siRNA duplexes targeting OCT3 (Hs_SLC22A3_3; Cat No: SI00721357) with Hiperfect transfection reagent and serum-free, antibiotic-free DMEM according to manufacturer’s recommendations (Qiagen, Valencia, CA, USA). After 48h following transfection, media were changed to DMEM containing 1% FBS and cells were treated with either metformin or phenformin.

**Statistics**

When indicated, the data were analyzed using Student’s t test with the Prism 6.0 biostatistics program (GraphPad Software). Data are presented as mean ± S.E.M.
Chapter (3)

Results

*Phenformin treatment significantly reduces OSCC cell viability*

To evaluate whether phenformin affects OSCC cell growth, dose-response cell viability assays were carried out. Initially, HN13 cell viability assays were conducted with cells cultured under high glucose conditions in 1% FBS DMEM. HN13 cells were treated with phenformin at doses ranging from 0 - 2 mM. After 3 days, MTS reagent was added to each well and cell viability was measured. As shown in Figure 2A, phenformin significantly reduced cell viability with doses ranging from 0.5 mM-2mM. Approximately 50% reduction in cell viability was achieved with 0.75mM phenformin. It has been suggested that a limiting factor affecting the interpretation of *in vitro* studies with metformin as an antineoplastic agent is the high concentration used to induce cellular responses. *In vitro* non-physiological conditions (i.e., presence of high glucose), which considerably differ from in vivo experimental models and clinical settings, may be responsible for the high doses of metformin needed to trigger responses in vitro [43,44].

Therefore, we decided to treat OSCC cells with phenformin in 1% FBS DMEM containing a more physiological glucose (5 mM) as compared to high glucose (25 mM). Interestingly, when HN6 and HN13 cells were treated under these conditions, a much lower dose of phenformin (10µM) was needed to significantly reduce OSCC cell viability by approximately 50% (Figure 2B). Because of these findings, we decided to choose 10µM phenformin as the appropriate dose for the following experiments.
Figure 2. (A) HN13 cells were plated in 10% FBS-containing high glucose (4.5g/L) DMEM in triplicate. After 24 hr, cell culture conditions were changed with 1% FBS high glucose DMEM with either vehicle control or phenformin at different doses. After 3 days, cell viability was measured. *p<0.05 vs. control. (B) Following a similar protocol in HN13 and HN6 cells, a much lower phenformin dose significantly affect cell viability when treated in low glucose (1 g/L) DMEM. *p<0.05 vs. control.
Phenformin activates AMPK signaling in an OCT independent manner

To gain preliminary insight into the role of OCTs in mediating the antineoplastic effects of phenformin, experiments were performed to examine whether AMPK signaling activation in response to phenformin was dependent on OCT function. First, we used a chemical inhibition approach by using an OCT pan-inhibitor such as quinidine. As shown in Figure 3A, AMPK activation in HN6 cells, as evidenced by ACC phosphorylation (pACC), was not prevented by the presence of quinidine at 10µM and 100µM. The phosphorylation of ACC is a downstream step in AMPK signal cascade and it is an indication of an activated AMPK [45]. In contrast, the phosphorylation status of ACC was downregulated to basal levels when quinidine–pretreated HN6 cells were treated with metformin (Figure 3B). These results suggested that phenformin, compared to metformin, might be inducing its intracellular effects in an OCT independent fashion.

To complement these observations, OCT3 was genetically knocked down in HN13 cells using short interfering RNA (siRNA) transfection. This was done to evaluate dependence of phenformin on OCT3 transport compared to metformin. HN13 cells were both transfected with a control siRNA or specific OCT3-targeted siRNA, and treated with either metformin or phenformin. Interestingly, phenformin-treated OCT3 siRNA targeted cells showed a stronger pACC expression compared to the control pACC. Marked reduction of OCT3 expression in those cells indicated a successful transfection. In contrast, in cells treated with metformin, there was a marked reduction of pACC expression in the OCT3 siRNA-targeted cells when compared to the control group. Collectively, these data indicated that metformin was not able to induce AMPK signaling activation in the absence of OCT3, which further validated its dependency on OCT-
mediated transport. Phenformin, however, induced AMPK activation in OCT3 depleted cells, strongly suggesting a lack of dependency on OCT transport (Figure 4).

![Table and images](image)

**Figure 3.** (A) In quinidine-treated HN6 cells, AMPK was still activated with 10µM phenformin as shown by phosphorylated ACC (pACC) levels. (B) However, signaling AMPK activation by metformin was prevented in the presence of quinidine.

![Table and images](image)

**Figure 4.** In genetically OCT3-knocked down HN13 cells, AMPK signaling activation (pACC) was evident in cells treated with phenformin in contrast to metformin.
Chapter (4)

Discussion

The biguanides metformin, phenformin and buformin are all derived from the plant *Galega officinalis* (French lilac) as depicted in Figure 5. These drugs were developed originally to treat hyperglycemia and type-2 diabetes. However, the use of the French lilac herb was commonly utilized to treat frequent urination and thirst in ancient Egypt and Europe [46]. In the 1920’s, biguanide was identified as the active ingredient of French lilac, and was further developed into therapeutics in the 1950s [18,47,48].

In the 1970s, the United States Food and Drug Administration withdrew phenformin from the market due to reports linking its use to fatal lactic acidosis in elderly patients.
with impaired renal function. Metformin (1,1 dimethylbiguanide), on the other hand, has shown a much more desirable safety profile and is currently considered the first-line drug of choice in managing non-insulin dependent type 2 diabetes. With more than 50 years of clinical use, this inexpensive, non-toxic and well-tolerated drug is used by approximately 120 million people worldwide [47].

The antineoplastic effects of metformin in oral carcinogenesis appear to be mediated by a direct action on tumor cells [41]. Significant benefits of the chronic administration of metformin have been demonstrated in mice by preventing the transformation of 4-nitroquinoline-1-oxide-induced oral premalignant, dysplastic lesions into OSCC tumors [49]. Since very limited impact was observed on serum components and metabolic markers including glucose, insulin and insulin growth factor-1, this study underscores the possibility of a direct effect at the site of OSCC development. Moreover, in a recent study conducted by Patel et al, it was reported that in human oral epithelial dysplasias and well-differentiated OSCC tumors, only OCT3 appeared to be highly expressed [41]. Conversely, undetectable or weak OCT3 expression was observed in less differentiated OSCC tumors, suggesting a previously unidentified link between the degree of tumor cell differentiation and the expression of OCT3. This study also showed that in OCT3-depleted OSCC cells, metformin was unable to induce AMPK activation and mTOR inhibition [41].

As it appears, phenformin may be a safer alternative than many commonly used chemotherapeutic agents when given to non-diabetic, closely monitored cancer patients. Although, metformin and phenformin are substrates for OCT1, the intrinsic OCT1 transport activity for metformin is 2-3 times higher than that for phenformin, implying
that phenformin, due to its more hydrophobic structure (Figure 6), may be less dependent on OCT mediated transport [50]. In fact, a recent study showed that OCT1 siRNA knockdown reduced sensitivity to metformin, but not to phenformin in epithelial ovarian cancer cell lines [51].

![Chemical structures of phenformin and metformin; phenformin is more hydrophobic due to the presence of a phenolic ring.](image)

**Figure 6.** Chemical structures of phenformin and metformin; phenformin is more hydrophobic due to the presence of a phenolic ring.

**Oncological use of phenformin**

A number of studies have suggested that phenformin may have anti-tumor properties. In 1968, Lugaro and Giannattasio described anticancer properties of biguanides [52]. Dilman and Anisimov, in 1979, showed in their study that phenformin acted as an adjunctive to cyclophosphamide antitumor effects in rats transplanted with squamous cell carcinoma and hepatoma-22a and Lewis lung tumor [53]. A year earlier, it was concluded that when taken orally, phenformin reduced 7,12-Dimethylbenzanthracene (DMBA)-induced mammary tumor development [54]. It was also found that in C3h/5N mice treated with
phenformin, the life span was extended by retarding cancer development and growth [55] and that phenformin showed inhibition of Elrich carcinoma in mice [56].

More recent studies have observed similar findings with regard to the antineoplastic effects of biguanides, and phenformin in particular. One study examined antineoplastic effects of phenformin on proliferation of cancer cell lines and concluded that phenformin exerts an inhibitory control on tumor cell growth and induces cell cycle arrest followed by apoptosis [28]. Another study found that phenformin significantly decreased the number of mitotic figures in tumor xenografts derived from two breast cancer cell lines (MCF-7 and MDA-MB-231) compared to untreated tumors. These effects may have also been associated with cell cycle arrest [57]. Indeed, consistent findings support phenformin’s role in cell cycle arrest in hepatoma Hep G2 cells [58], human aortic smooth muscle cells, rabbit aortic strip [59] and mouse embryonic fibroblasts [60]. Appleyard et al also reported that MCF-7 treated with phenformin showed a type of stromal growth non supportive of tumor progression[57].

Recently, the role of phenformin as an anti-neoplastic agent has been demonstrated acting on signaling pathways leading to tumor cell growth inhibition [25]. There are reports of formation of stroma in a mode of action that may be due to the formation of tumor stroma not supportive of tumor growth [61-64].

Phenformin is a 50-fold more potent inhibitor of mitochondrial complex I than metformin [65,66]. Moreover, uptake of metformin, but not phenformin, into tissue appears to require the expression of at least OCT1, which is highly expressed in hepatocytes [35]. Compared to metformin, and due to its greater potency and broader tissue bioavailability,
phenformin has been shown to delay tumor progression in a Pten+/− spontaneous lymphoma mouse model to a much greater extent than metformin [25].

Our findings with regard to phenformin ability to interfere with mTOR pathway and cause cancer cell regression are in agreement with the above-mentioned studies. In HN6 and HN13 cell viability assays, for example, a marked reduction in cancer cell population was evident when treated with phenformin. In fact, a near 50% cell viability reduction, compared to control group, was evident at relatively low concentrations of phenformin especially under low glucose media conditions (5.5mM).

To elucidate whether OCTs are required to facilitate intracellular transport of phenformin and activate AMPK signaling pathway, a chemical and a genetic inhibition of OCTs studies were conducted. Quinidine, a chemical pan-inhibitor of OCTs, was used on HN6 cells prior to treatment with phenformin. In our findings, AMPK signaling pathway was activated (evident by pACC) in HN6 cells treated with phenformin despite chemical inhibition of OCTs. However, in samples treated with metformin, there was no evidence of AMPK signaling pathway activation. This suggests that phenformin activates AMPK signaling in an OCT independent manner whereas metformin appears to rely on OCT transport.

To further test phenformin’s dependency on OCT transport, genetic inhibition of OCTs (OCT3 specifically) was carried out on HN13 cells using short interference RNA. Samples were treated either metformin or phenformin. Upon confirmed successful transfection indicated by marked reduction of OCT3 expression, we found that phenformin induced AMPK activation in OCT3 depleted cells, strongly suggesting a lack
of dependency on OCT transport. In contrast, in cells treated with metformin, there was a marked reduction of pACC expression in the OCT3 siRNA-targeted cells when compared to the control group. Collectively, these data indicated that metformin was not able to induce AMPK signaling activation in the absence of OCT3, which further validated its dependency on OCT-mediated transport. Phenformin, however, appears to cause activation AMPK signaling pathway in a manner that is OCT-independent.

**Effect of glucose concentration in cell culturing media**

The cell viability assays in our study demonstrated that phenformin is noticeably effective in reducing cell viability at relatively lower doses. In medium containing high glucose concentration (4.5g/L or 25mM), 0.75 mM phenformin reduced HN13 cell viability by approximately 50% when compared to control. It is important to mention that glucose concentration of 25 mM is about 5-times higher than the physiological blood levels in humans [67]. The amount of glucose in cell culture formulations ranges from 5.5 mM to as high as 55 mM. Many commercially-available media formulations are supplemented with approximately 5.5 mM D-glucose which approximates normal blood glucose levels *in vivo* [68]. Therefore, HN6 and HN13 cell viability assays in response to a range of phenformin doses were again conducted in cell culture conditions that mimicked a more physiologic media of 5.5 mM D-glucose. In fact, a recent study concluded that cancer cells exhibit diverse responses to glucose limitation in vitro, and underscores the importance of taking into account the glucose concentration when evaluating the effects of biguanides on cancer cells [69]. Our findings were in agreement with this previous study. When HN13 cells were treated with various concentrations of phenformin ranging from 0-2mM in high glucose media, about 50% cell death occurred.
at a concentration of 0.75 mM phenformin. When the viability assays were repeated in low glucose media, approximately 50% cell death in HN13 and HN6 were observed with 10µM phenformin, a 75-fold reduction in the concentration originally used in the high glucose experiments to reach a similar decrease in cell viability. This is consistent with previous findings, and does underscore the dependence of cell culturing conditions for the appropriate effectiveness of biguanides, in this case phenformin, for their actions in in vitro experiments.
Conclusion

The primary goal of this study was to determine whether the biguanide phenformin impacts OSCC cell viability, and causes the activation of the AMPK pathway in an OCT mediated manner. Unlike metformin, phenformin-mediated AMPK signaling activation in OSCC cell lines HN6 and HN13 appears to take place in a manner that is independent of OCT mediated transport. In our study this was partly evident from in vitro experiments that target OCTs both chemically, through the use of quinidine, as well as genetically through RNA interference. This could be attributed to the hydrophobic structure of phenformin, which may allow for more passive diffusion into the cell rather than an active transport mechanism. Further studies are needed to explore the ability of phenformin to activate the AMPK pathway in different OSCC cell lines expressing different OCTs. Moreover, it would be important to elucidate the role of phenformin and AMPK signaling on OSCC cell viability. In fact, further studies may include in vivo models to elucidate the antineoplastic properties of phenformin in genetically OCT deficient mice. Once it is established that phenformin is indeed more efficient to metformin as an antineoplastic agent for it exerts its action in a relatively lower concentrations and in an apparent OCT independent manner, it could be incorporated in head and neck/oral cancer therapy as an adjunct to conventional chemotherapeutic agents.
References


