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Efficacious Anti-Cancer Drugs Targeting Nicotinamide *N*-Methyltransferase (NNMT) in Cultured Human Oral Squamous Cell Carcinoma (OSCC)

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Abstract

Oral squamous cell carcinoma (OSCC) is major cause of human cancer. The enzyme, nicotinamide *N*-methyltransferase (NNMT) is overexpressed in a variety of human cancers including OSCC. Our laboratory identified two small molecule inhibitors of NNMT (AG-670 and AO-022) based on a pharmacophore of the *in silico* nicotinamide binding site. These inhibitors were demonstrated to inhibit the isolated NNMT enzyme with EC_{50} values in the micromolar range. Using an oral squamous cancer cell line as a model, it was demonstrated that NNMT is expressed in SCC-4 cells, but not MCF 7 cells, and that the inhibitors were cytotoxic to SCC-4 cells (IC_{50} values in the micromolar range). In an endeavour to investigate the mechanism behind their lethality, sub-lethal doses of the inhibitors were demonstrated to inhibit *in situ* mitochondrial oxygen consumption in SCC-4 cells but not in MCF 7 cells. It was further demonstrated that the NNMT inhibitors did not directly inhibit mitochondrial electron transport chain activity. Thus, we can deduce that the NNMT inhibitors effect mitochondrial activity indirectly via NNMT. We conclude that NNMT is a potential drug target for oral cancer.

Keywords: oral squamous cell carcinoma; nicotinamide *N*-methyltransferase; mitochondria; cellular oxygen consumption; SCC-4; MCF 7; AG-670; AO-022

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for up to 90% of all oral malignancies and is the sixth most common cancer worldwide [1]. Treatment involves surgical removal of the tumour, radiation, and chemotherapy although such treatments are not always satisfactory with 5-year survival of only 50% being recorded [1,2]. We were interested in a more targeted approach to compliment the established cancer therapeutics. The enzyme, *N*-nicotinamide methyltransferase (NNMT) is overexpressed in a variety of human cancers, including oral squamous cancer biopsies [3] and has recently been investigated by several laboratories as a potential anti-cancer target [4–11]. Nicotinamide *N*-methyltransferase (NNMT) catalyses the methylation of nicotinamide (NAM) by transferring the methyl group the methyl donor S-adenosyl-L-methionine to nicotinamide, subsequently generating S-adenosyl-L-homocysteine, and 1-methylnictotinamide [12,13] (Figure 1A). NNMT thus modulates intracellular NAD^+ levels, which are crucial for energy metabolism,

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oxidative phosphorylation [14,15] and metabolic enzyme activity via NAD^+-dependent sirtuin deacetylases [16].

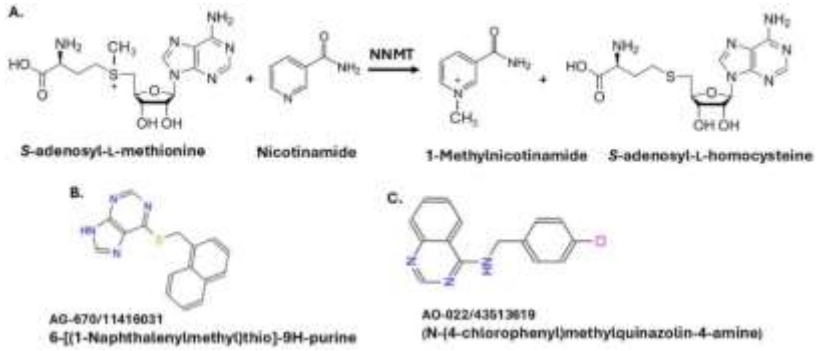

Figure 1. Depiction of the enzyme activity catalyzed by Nicotinamide *N*-Methyltransferase (NNMT) and representative structures of the NNMT inhibitors (AG-670 & AO-022) investigated. (A) Nicotinamide *N*-methyltransferase (NNMT) is a one-carbon group cytosolic enzyme that is involved in the catalysis of methylation, by S-adenosyl-L-methionine, of nicotinamide thus generating S-adenosyl-L-homocysteine and 1-methylnictotinamide [12,13]. The two small molecule inhibitors of NNMT (B) AG-670/11416031 (AG-670) and (C) AO-022/43513619 (AO-022) were selected based on *in silico* analysis defining the pharmacophore of the nicotinamide binding site of NNMT [Supplementary Section 1].

Under normal physiologic conditions, NNMT is predominantly expressed by hepatocytes in the liver, white adipose tissue, skeletal muscle and lung, while multiple mesenchymal cell types have lower levels of NNMT expression [17,18]. However, numerous studies have suggested that NNMT expression is significantly increased in several kinds of cancer, including neuroblastoma [19], oral squamous cell carcinoma [3], papillary thyroid cancer [20], lung cancer [21], breast cancer [22], gastric cancer [23], pancreatic cancer [24], colorectal cancer [25], renal carcinoma [26], and ovarian clear cell carcinoma [27]. High NNMT expression in these cancers appears to be inversely associated with the tumour size and progression, suggesting that NNMT may have potential effects in an initial step of malignant conversion [28]. However, and interestingly, there are also cancer cell lines where NNMT is not expressed such as in the estrogen-receptor positive breast cancer cell line MCF 7 [29,30].

Several NNMT inhibitors have been identified to date, such as methylated quinolines [6] nicotinamide analogs [7,8], covalent inhibitors [4,9], and amino-adenosine and alkynyl derived bi-substrate inhibitors [5,10,11]. Our laboratory identified two small molecule inhibitors of NNMT (AG-670 and AO-022) [Figure 1B,C] based on a pharmacophore of the *in silico* nicotinamide binding site [Supplementary section 1]. The effect of these modulators of NNMT on OSCC cell viability and energy metabolism was evaluated.

2. Results

The enzyme, nicotinamide *N*-methyltransferase (NNMT) is overexpressed in a variety of human cancers. Figure 2 demonstrates that NNMT is expressed in Hs578T cells (a triple negative breast cancer cell line), SCC-4 cells (an oral squamous cell carcinoma cell line), DOK cells (a pre-cancerous dysplastic oral keratinocytes cell line) and primary oral gingival keratinocytes (PGK cells). However, Figure 2 demonstrates that NNMT is not expressed in estrogen (ER), progesterone (PR), and glucocorticoid receptor-positive MCF7 cells, confirming data presented by others [29,30].

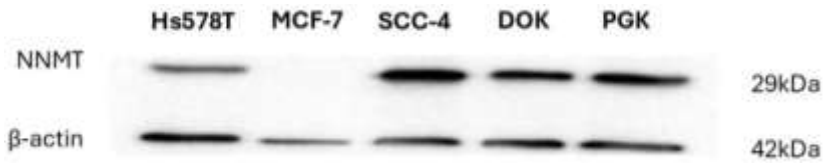

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Figure 2. Immunoblot of Nicotinamide *N*-Methyltransferase (NNMT) expression in various cultured cells. NNMT is expressed in Hs578T cells (a triple negative breast cancer cell line), SCC-4 cells (an oral squamous cell carcinoma cell line), DOK cells (a pre-cancerous dysplastic oral keratinocytes cell line) and primary oral gingival keratinocytes (PGK cells). NNMT is not expressed in the estrogen (ER), progesterone (PR), and glucocorticoid receptor-positive MCF7 cells.

Figure 3 demonstrates the potency of the two small molecule inhibitors (AG-670 and AO-022) on isolated NNMT enzyme activity and SSC4 cell cytotoxicity. Figure 3A,B demonstrate that AG-670 and AO-022 directly inhibits enzyme activity with EC_{50} values of 2.7μM (95% CI: 1.4 – 5.8) and 37.5μM (95% CI: 23.4 - 60.5), respectively. Figure 3C,D demonstrate that AG-670 and AO-022 are potent to SCC-4 cells with IC_{50} values of 41.8μM (95% CI: 33.5 - 65.3) and 154.9μM (95% CI: 108.5 – 268.4), respectively.

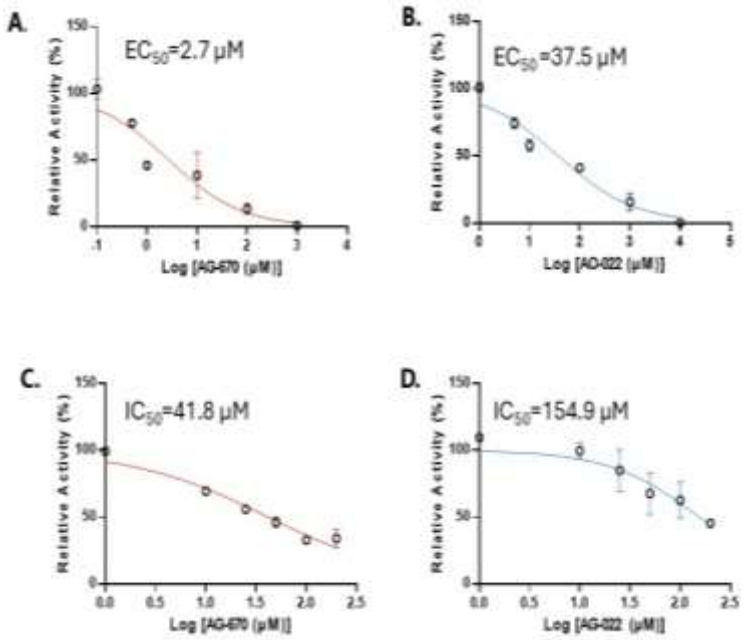

Figure 3. The Effect of AG-670 and AO-022 on nicotinamide *N*-methyltransferase (NNMT) isolated enzyme activity (EC_{50}) & SCC-4 cell cytotoxicity (IC_{50}). (A & B) An NNMT Inhibitor Screening Assay kit (Sigma-Aldrich) was used to determine potency of potential NNMT inhibitors (AG-670 and AO-022) on the isolated enzyme (EC_{50}). (C & D) SCC-4 cell viability was determined by Alamar blue assay using a range of NNMT inhibitor concentrations over 72h using 1% FBS (IC_{50}).

We next determined the effect of sub-lethal doses of NNMT inhibitors (AG-670 & AO-022) on oxygen consumption rates (OCR) in SCC-4 cells (Figure 4), cells demonstrated to express NNMT (Figure 2). Cellular oxygen consumption rates are regularly used as an index of general cellular metabolism in primary cells and in many, but not all, cancer cells. We could demonstrate that both AG-670 & AO-022, at a sublethal dose of 10μM, inhibit cellular oxygen consumption (Figure 4A) by inhibiting *in situ* mitochondrial oxygen consumption (~3-fold) in SCC-4 cells (Figure 4B). Similar results were observed for Hs578T cells (Figure S4) and human dysplastic oral keratinocyte (DOK) cells (not shown), both of which express NNMT (Figure 2)

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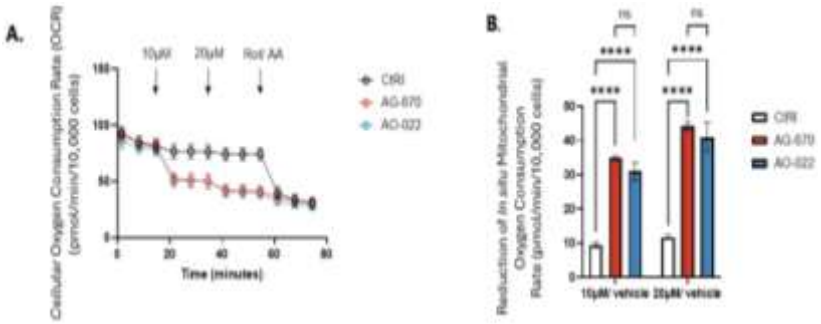

Figure 4. Effect of sublethal doses of NNMT inhibitors (AG-670 & AO-022) on Oxygen Consumption Rates (OCR) in SCC-4 cells. (A) Cellular and (B) reduced *in situ* mitochondrial oxygen consumption (OCR) in SCC-4 cells was determined by Seahorse XF analyser (Agilent) in the absence and presence of 10μM AG-670 & AO-022. The control was addition of vehicle (DMSO) alone (CtRl). Antimycin A (AntiA) and rotenone (Rot) were ultimately added to inhibit *in situ* mitochondrial oxygen consumption in order to quantify the cellular oxygen due to mitochondrial activity.

As the NNMT inhibitors were designed for the nicotinamide site in NNMT, it was decided to investigate the efficacy of AG-670 & AO-022 on oxygen consumption by isolated mitochondrial (Figure 5). It was demonstrated that neither inhibitor effected oxygen consumption in isolated mitochondria respiring on glutamate and malate which are as a source of matrix $NADH_2$ for complex 1 initiated electron transport chain activity.

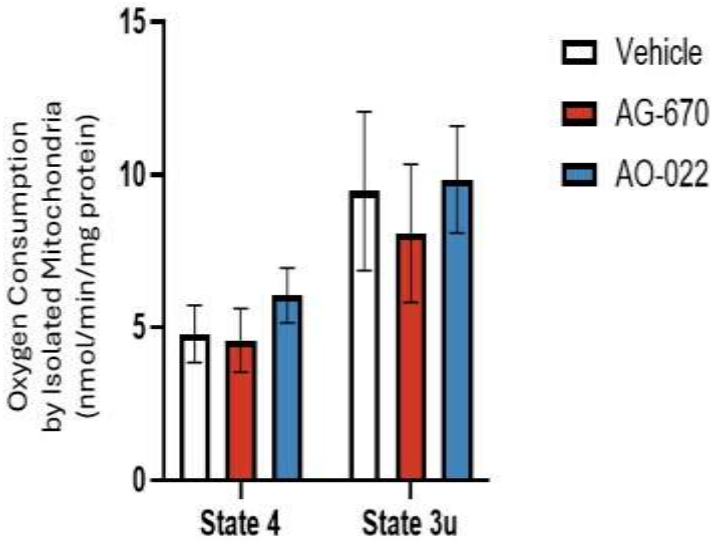

Figure 5. No Effect of NNMT inhibitors (AG-670 & AO-022) on Isolated Mitochondrial activity. Oxygen consumption by isolated mitochondria from rat liver using NADH-dependent substrates (glutamate and malate) in state 4 and state 3 uncoupled rate (an index of maximal electron transport chain activity) conditions, were measured the presence and absence of 10μM NNMT inhibitors AG-670 & AO-022.

We then determined the effect of sub-lethal doses of NNMT inhibitors (AG-670 & AO-022) on oxygen consumption rates (OCR) in MCF 7 cells (Figure 6), cells demonstrated not to express NNMT (Figure 2). We demonstrated that neither AG-670 or AO-022, at a sublethal dose of 10μM, inhibited cellular oxygen consumption (Figure 6A) or *in situ* mitochondrial oxygen consumption in MCF 7 cells (Figure 6B).

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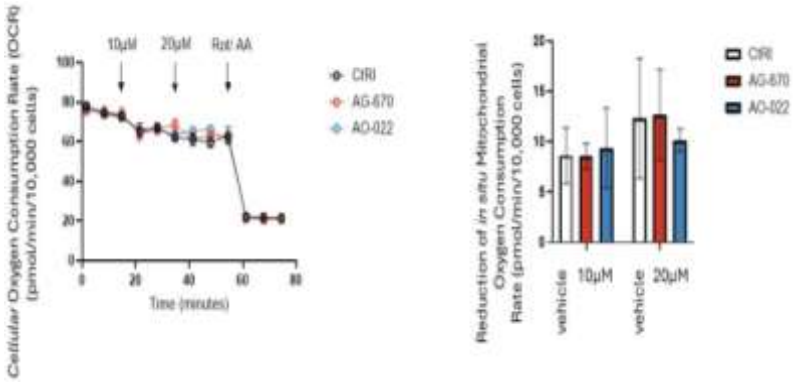

Figure 6. Effect of sublethal doses of NNMT inhibitors (AG-670 & AO-022) on Oxygen Consumption Rates (OCR) in MCF 7 cells. (A) Cellular and (B) reduced *in situ* mitochondrial oxygen consumption (OCR) in MCF 7 cells was determined by Seahorse XF analyser (Agilent) in the absence and presence of 10μM AG-670 & AO-022. The control was addition of vehicle (DMSO) alone (CtRl). Antimycin A (AntiA) and rotenone (Rot) were ultimately added to inhibit *in situ* mitochondrial oxygen consumption in order to quantify the cellular oxygen due to mitochondria.

3. Methods

3.1. In Silico Modelling

The two small molecule inhibitors of NNMT (AG-670 and AO-022) [Figure 1B,C] were purchased from Specs Compound Handling B.V./eMolecules (Zoetermeer, The Netherlands) based on our work defining the pharmacophore of the *in silico* nicotinamide binding site [Supplementary Information 1].

3.2. Cell Culture

All cells were purchased from the American Type Culture Collection (ATCC) and cultured as per their recommendations. SCC-4 cells were originally derived from stage 3 tongue cancer in a 50 year old male [31]. MCF-7 cells are a human breast cancer cell line derived in from a 69-year-old female with metastatic adenocarcinoma. It is a key Luminal A subtype cell line, characterized by being estrogen (ER), progesterone (PR), and glucocorticoid receptor-positive [32]. Hs578T is a triple negative (no estrogen, progesterone and HER2 receptors) breast cancer cell line originally from epithelial cells isolated from breast tissue derived from a 74-year-old female breast cancer patient [33,34]. Dysplastic oral keratinocyte (DOK) cells (originally from the dorsal part of the tongue of a 57 year old man) and primary gingival keratinocyte (PGK) cells were also cultured. All cells were cultured as described in Karavyraki & Porter [35].

3.3. Immunoblotting

Detection of NNMT was assessed by immunoblot using Novus Biologicals antibody NBP2-00537 with cell lysates, using methodology described in Ge *et al.* [36].

3.4. Cytotoxicity (IC_{50})

Cell viability was determined by Alamar blue assay using a range of NNMT inhibitor concentrations over 72h using 1% FBS.

3.5. Enzyme Activity (EC_{50})

An NNMT inhibitor screening assay kit (Sigma-Aldrich) was used to determine potency of inhibitors on the isolated enzyme.

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3.6. Oxygen Consumption Rates (OCR)

Cellular and *in situ* mitochondrial oxygen consumption (OCR) were determined by Seahorse XF analyser (Agilent) as described in Karavyraki & Porter [35]. Oxygen consumption by isolated mitochondria from rat liver using NADH-dependent substrates (glutamate and malate) in the presence and absence of NNMT inhibitors was determined using a Rank Oxygen Electrode as described in Martin *et al.* [37].

4. Conclusions

Oral squamous cell carcinoma (OSCC) accounts for up to 90% of all oral malignancies and is the sixth most common cancer worldwide. Our research was interested in finding molecular targets to treat this cancer. The enzyme, *N*-nicotinamide methyltransferase (NNMT) is one such candidate and has been shown to be overexpressed in a variety of human cancers [3,20–27]. Hence NNMT has been investigated by several laboratories as a potential anti-cancer target [4–11]. In this study, NNMT is shown to be expressed in oral squamous cell carcinoma (SCC-4) cells, dysplastic oral keratinocyte (DOK) cells, primary gingival keratinocyte (PGK) cells and the triple negative breast cancer cell line Hs578T. These data are consistent with NNMT expression in OSCC biopsies [3]. Furthermore, we confirm that NNMT is not expressed in MCF 7 breast cancer cells, as has been demonstrated by others [29,30]. In addition, the NNMT inhibitors selected from our pharmacophore design (Supplementary section 1) have been demonstrated to be direct inhibitors of the enzyme NNMT and cytotoxic to SCC-4 cells. In an endeavour to examine the mechanism behind that potency, AG-670 and AO-022 were tested for efficacy on cellular oxygen consumption, an index of overall metabolism in cells. It was demonstrated that sublethal doses of AG-670 and AO-022 inhibit *in situ* mitochondrial oxygen consumption in intact SCC-4 (and DOK and Hs578T cells) from which we deduce that a restriction of ATP supply is a prelude to, and probable factor in, the potency of the drugs. Furthermore, although the drugs were designed to target NNMT, it was important to establish whether the inhibition of *in situ* mitochondrial oxygen consumption in SCC-4 cells might be due to a direct effect on mitochondrial function independent of NNMT. This was addressed firstly, by demonstrating that AG-670 and AO-022 do not directly inhibit mitochondrial electron transport chain function in isolated mitochondria and secondly, by demonstrating that AG-670 and AO-022 had no effect on *in situ* mitochondrial oxygen consumption in MCF 7 cells, *i.e.* cells that don't express NNMT. We therefore conclude that NNMT activity directly effects mitochondrial activity *in situ* and that the inhibitors of NNMT inhibit *in situ* mitochondrial oxygen consumption in SCC-4 indirectly via inhibition of NNMT. Possible explanations as to the mechanism behind the indirect inhibition of mitochondrial function via NNMT stem from a couple of interesting observations in the literature. Parsons *et al.* [38] revealed that NNMT expression contributes to cell survival by enhancing complex I activity, a process that appears to be mediated via the protection of the mitochondrial complex 1 subunit, NDUFS3, from degradation. The same group also demonstrated that these effects arise due to increased production of MNA, as well as demonstrating that NNMT and 1-methylnicotinamide are cytoprotective against Complex I inhibitors MPP^+ and rotenone, which is mediated via the maintenance of Complex I activity arising from the protection of NDUFS3 from inhibitor-mediated damage. In the SH-SY5Y cell line, expression of NNMT substantially reduced cell death, which correlated with an increase in the ATP/ADP ratio and Complex I activity. Interestingly, Liu *et al.* [39] demonstrated that NNMT increases mitochondrial complex I activity *in situ* in SH-SY5Y cells via sirtuin 3, a mitochondrial NAD^+-dependent deacetylase [40,41]. In conclusion, our study has demonstrated that NNMT is a potential drug target for oral cancer.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

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Conflicts of Interest: The authors declare no conflict of interest.

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