Kinomic profile in patient-derived glioma cells during hypoxia reveals c-MET-PI3K dependency for adaptation

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Introduction

Hypoxia is common in the tumour microenvironment (TME) due to a difference in supply and demand leading to the expression of hypoxia induced factors (HIFs) that leads to enhanced tumour survival (transition from oxidative to glycolytic metabolism and altered drug response[1]).

Glioblastoma (GBM) is an often fatal form of brain cancer that has a high level of resistance to therapy and commonly recurs. This is due to intratumoral hypoxia that is common in GBM. The hypoxia contributes to the maintenance of the glioma-like stem cells (GSCs) by supporting multipotency, self renewal, and tumorigenicity[2]. Hypoxia also induces migration and invasiveness of GBM cells[3]. Yet, hypoxia is not considered in many drug screening designs.

Hypoxia disrupts cellular kinome network to increase the robustness and resilience of oncogenic pathways. Through targeting hypoxic-dependent kinase signalling hubs, it is possible to prevent resistance and improve patient outcomes.

Aim

To identify hypoxia-protein kinase pathways that can be targeted by current novel therapies using a **bench to bedside and back** approach.

Methods

Cell Cultures
Samples of real patients' GBM GPC cells from National Neuroscience Institute Singapore (NNI) are cultured.

Kinase Inhibitor Screen and Viability Assay
Cultured cells are treated with various kinase inhibitors in either normoxic or hypoxic conditions, and then stained to assess extent of cytotoxicity.

Kinase-kinase pathway analysis
For the more efficacious kinase inhibitors in hypoxic conditions, their respective drug targets are mapped and analysed to identify key pathways involved in the hypoxic adaptation of GBM cells.

Unweighted ranking analysis
Each kinase was scored based on:
1. Direct interaction with HIF-1α
2. Network Topology
3. Correlation of gene expression with patients' overall survival, based on publicly available genetic data repositories

Pharmacological analysis
Further pharmacological analysis was conducted based on available dose-response curves, analysis of drug-drug interactions, as well as the effects of combination therapies.

Bioinformatics analysis
Using public GBM databases, differential gene expression analysis was conducted on various tumours. Transcription factor enrichment analysis was conducted on genes that were significantly upregulated. Gene data was then mapped to specific protein kinases.

Statistical analysis
A mixed model ANOVA was used to analyse dependent variables with mixed measures. A two-way ANOVA was used for dependent variables with two main factors. All other variables were analysed with one way ANOVA.

Additional Experimentation

siRNA Knockdown
For additional investigation, additional GPC cells were transfected with siRNA that targets c-MET. To analyse Off-target effects of these inhibitor, siRNA exposed GPC cells were treated with c-MET inhibitors and examined for viability.

Immunoblotting
Cells were lysed and far-infrared immunoblotting conducted to quantify protein bands, to investigate the effect on α-tubulin polymerization.

Animal experiments
Cells from NNI were implanted into mice for a tumour to form. The mice were treated with various drugs and kinase inhibitors, and the tumour weight analysed.

Results

Determination of c-MET-PI3K Hypoxic Response Pathway

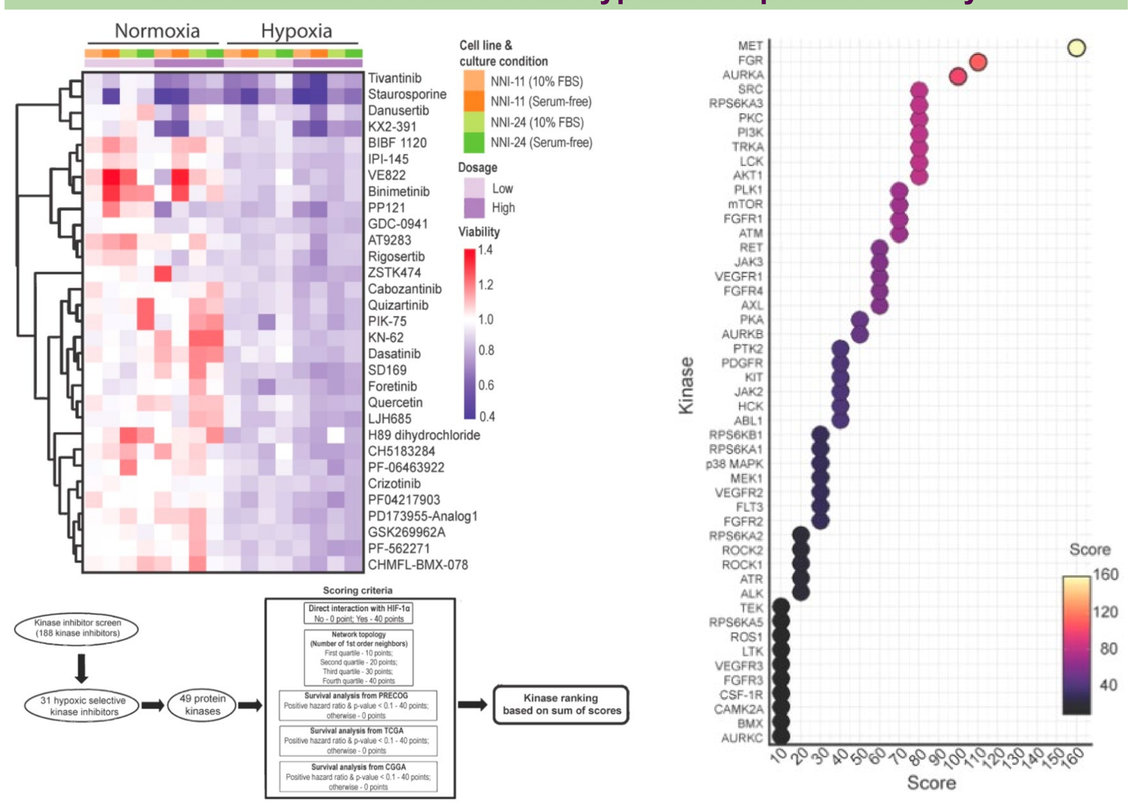

Figure 1. Kinomic Landscape in Hypoxic Adaptation of GPCs. (Left, Top) Selected kinase inhibitors with hypoxia-selective cytotoxicity in GPCs. Blue color indicates reduced viability, while red color indicates increased viability compared to DMSO-treated cells. (Left, Bottom) Workflow and scoring criteria used in the unweighted ranking analysis. Protein kinases are ranked based on the connectivity network and the correlation between their expressions and GBM patients' overall survival. (Right) Kinase ranking based on the unweighted sum of scores of each protein kinase. Higher ranks predict a more important role of the kinase in tumor hypoxia of GPCs based on the network analysis and clinical data.

Only 15% of inhibitors were cytotoxic in hypoxia meanwhile only 2.7% of inhibitors were cytotoxic to glioma-propagating cells (GPCs) in normoxia and hypoxia. The screen also revealed 31 hypoxic selective inhibitors that can be crucial in GPC hypoxic response. The drug targets were then mapped out to 49 protein kinases, with c-Met ranking the top and sharing mutual interaction with PI3K/Akt kinase.

The kinase inhibitor screen revealed a substantial modifying effect of oxygen environment on the effectiveness of small molecule inhibitors. The blockade of c-MET signaling successfully weakened GPCs, resulting in increased apoptosis.

c-MET Dependent Hypoxic Response Pathway

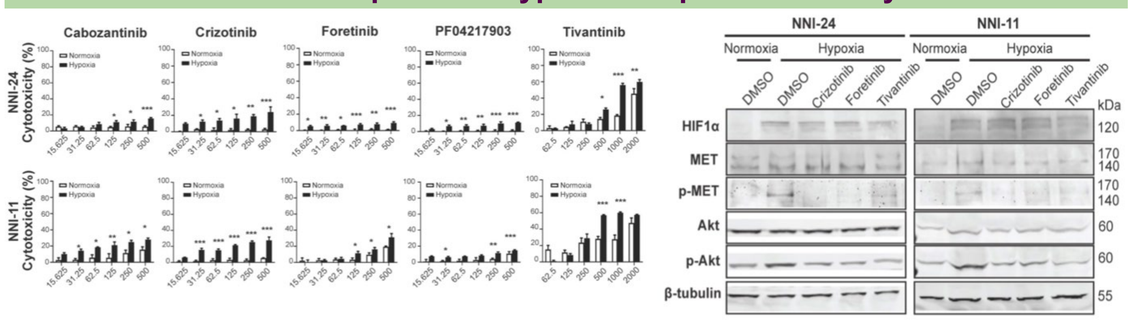

Figure 2. c-MET-dependent hypoxic response is mediated by PI3K/Akt pathway. (Left) Cell viability assays of NNI-24 and NNI-11 treated with increasing concentrations of c-MET inhibitors and exposed to normoxic and hypoxic conditions. (Right) Representative immunoblots of indicated proteins in NNI-24 and NNI-11 treated with crizotinib (500 nM), foretinib (500 nM) and tivantinib (1000 nM) and exposed to hypoxia. β-tubulin which serves as a loading control was from the same samples.

Conclusion

Tumour Hypoxia is an important aspect of the TME and should be considered as a factor in the preclinical testing of potential cancer drugs.

This research further substantiates the use of the **"bench to bedside and back"** approach in preclinical drug testing to improve the accuracy and validity of the results.

The cytotoxicity effects of c-MET inhibitors were markedly increased under hypoxia. Hypoxia enhances the phosphorylation of c-MET following HIF-1 upregulation which is effectively diminished by c-MET inhibitors.

The kinase interaction mapping suggests that Src and PI3K/Akt are key downstream signalling hubs of c-MET activation. Exposure to hypoxia also increased cytotoxicity of PI3K inhibitors in a dose dependent manner.

PI3K/Akt mitigates oxidative stress caused by metabolic reprogramming in hypoxia. Therefore, inhibition under hypoxia causes increased apoptosis as a result of oxidative catastrophe.

Synergy between c-MET Inhibitors with Temozolomide (TMZ) under Hypoxia

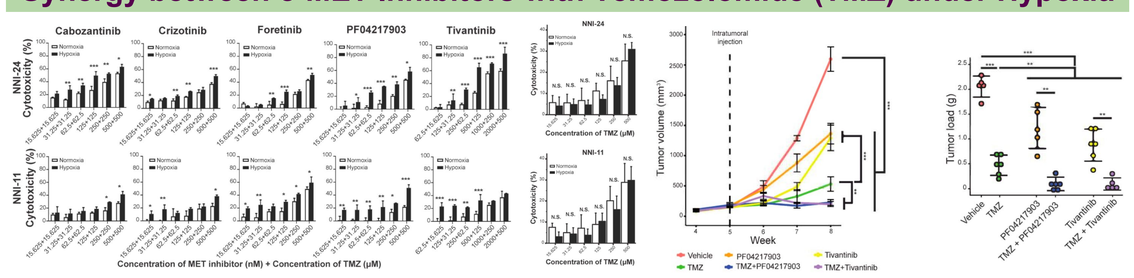

Figure 3. Hypoxia-dependent drug synergism between c-MET inhibitors and TMZ show enhanced anti-tumor effect in mouse GPC xenografts. (Left-Middle) Cell viability assays of NNI-24 and NNI-11 treated with combined therapy of c-MET inhibitors and TMZ (Left) or TMZ alone (Middle) at increasing concentrations and exposed to normoxic and hypoxic conditions. (Right) Growth of GPC xenografts in mice treated with vehicle, TMZ, PF04217903, PF04217903+TMZ via intratumoral injection and tumor load of GPC xenografts in mice treated with vehicle, TMZ, tivantinib, tivantinib+TMZ via intratumoral injection.

TMZ is the current standard of care chemotherapeutic drug for newly diagnosed GBM patients. Hypoxic selectivity of c-MET inhibitors was preserved even in combination with TMZ in a dose dependent trend. Among the five c-MET inhibitors, PF04217903 (PF) and tivantinib exhibited the most notable drug synergism.

Drug synergism was also preserved in vivo. PF and tivantinib induced activation of caspase-3 in the hypoxic regions, resulting in notable apoptosis in both hypoxic and normoxic conditions. This suggests that c-MET fortified TMZ cytotoxicity in the hypoxic environment.

Discussion

This therapeutic synergy demonstrates why targeting c-MET sensitizes GBM to the cytotoxic effect of TMZ and potentiates the overall treatment outcome, making c-MET a promising adjuvant candidate for GBM therapy.

c-MET inhibitors adversely disrupts tumour proliferation, neovascularisation and distal metastasis across different cancer types including gastric, breast, hepatic, pancreatic and lung carcinomas. They lead to an accumulation of intracellular ROS in GPCs that outstripped the endogenous antioxidant mechanism resulting in apoptotic cell death in a hypoxic-dependent manner. The inhibitors also prevent long term survival mechanisms such as angiogenesis and epithelial-to-mesenchymal transition.

This method of combining a kinase inhibitor screen, network analysis, and an unweighted scoring algorithm that incorporates clinical data helps to screen for the potential effectiveness on the different drugs on a wide variety of cancers. It can also help identify common gene targets that can be targeted for novel treatments.

The changing kinomic landscape and kinase inhibitor responses advocate an overhaul of cancer drug development. By taking into account factors and conditions such as tumour hypoxia, this method allows existing treatments to be further enhanced in a real-world context, leading to better patient outcomes.

References

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