Phenformin Inhibits Myeloid-Derived Suppressor Cells and Enhances the Anti-Tumor Activity of PD-1 Blockade in Melanoma

Sun Hye Kim[1,4], Man Li[1,4], Sebastian Trousil[1], Yaqing Zhang[2], Marina Pasca di Magliano[2], Kenneth D. Swanson[3] and Bin Zheng[1]

Biguanides, such as the diabetes therapeutics metformin and phenformin, have shown antitumor activity both in vitro and in vivo. However, their potential effects on the tumor microenvironment are largely unknown. Here we report that phenformin selectively inhibits granulocytic myeloid-derived suppressor cells in spleens of tumor-bearing mice and ex vivo. Phenformin induces production of reactive oxygen species in granulocytic myeloid-derived suppressor cells, whereas the antioxidant N-acetylcysteine attenuates the inhibitory effects of phenformin. Co-treatment of phenformin enhances the effect of anti-PD-1 antibody therapy on inhibiting tumor growth in the BRAF V600E/PTEN-null melanoma mouse model. Combination of phenformin and anti PD-1 cooperatively induces $CD8^+$ T-cell infiltration and decreases levels of proteins that are critical for immune suppressive activities of myeloid-derived suppressor cells. Our findings show a selective, inhibitory effect of phenformin on granulocytic myeloid-derived suppressor cell–driven immune suppression and support that phenformin improves the anti-tumor activity of PD-1 blockade immunotherapy in melanoma.

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INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are one of the major types of immune cells that contribute to tumor-induced immune suppression and escape from immune elimination (Marvel and Gabrilovich, 2015). They can be broadly divided into two main populations, granulocytic (G-) (also known as polymorphonuclear) and monocytic (M-) MDSCs, which are characterized by $CD11b^+Ly6G^{hi}Ly6C^{int}$ and $CD11b^+Ly6G^{lo}Ly6C^{hi}$, respectively, in mouse models (Marvel and Gabrilovich, 2015). MDSCs exert potent immunosuppressive activities toward T cells through multiple mechanisms, including expression of arginase 1, inducible nitric oxide synthase, and release of reactive oxygen species (ROS) (Marvel and Gabrilovich, 2015). Given their prominent role in tumor immune evasion, targeting MDSC-mediated immune suppression has been suggested to be an attractive approach to modulate tumor immunity for treating cancers (Draghiciu et al., 2015; Marvel and Gabrilovich, 2015).

Biguanides, such as metformin and phenformin, are commonly used to treat type 2 diabetes. Treatment with metformin in patients with type 2 diabetes has been found to be associated with lower cancer risks and lower cancer-related mortality rates (Morales and Morris, 2015). Biguanides inhibit mitochondrial complex 1 of the respiratory chain, which increases the adenosine monophosphate (AMP) to adenosine triphosphate ratio, resulting in AMP-activated protein kinase (AMPK) activation, which has been proposed to be a major mediator of their anti-tumor activity (Hardie et al., 2015; Pollak, 2013; Wheaton et al., 2014). However, most studies on the anti-tumor activities of biguanides have been focused on their cell-autonomous effects on cancer cells, but their potential effects on immune cells within the host and tumor microenvironment have remained largely unexplored.

We previously investigated the anti-tumor effect of biguanide AMPK activators in combination with BRAF inhibitors in various preclinical melanoma models (Shen et al., 2013; Zheng et al., 2009). These studies showed that phenformin, but not metformin, enhanced the efficacy of BRAF inhibitors by inhibiting the proliferation of BRAF-mutated melanoma cells in vitro and suppressing BRAF-driven tumor growth in mouse models (Yuan et al., 2013). More recently, we also found that phenformin and the extracellular signal-regulated kinase inhibitor SCH772984 exhibited synergistic anti-proliferative activities in NF1-mutant melanoma cells (Trousil et al.,

[1]*Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, USA;* [2]*Department of Surgery, University of Michigan, Ann Arbor, Michigan, USA; and* [3]*Department of Neurology, Brain Tumor Unit, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA*

[4]*These authors contributed equally to this work.*

Correspondence: Bin Zheng, Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School Building, 13th Street, Room 3013, Charlestown, Massachusetts 02129, USA. E-mail: bin.zheng@cbrc2.mgh.harvard.edu

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMP, adenosine monophosphate; AMPK, adenosine monophosphate-activated protein kinase; CFSE, carboxyfluorescein succinimidyl ester; G-MDSC, granulocytic myeloid-derived suppressor cell; MDSC, myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; ROS, reactive oxygen species

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2017). In this study, we examine the effects of phenformin on immune cells in the tumor microenvironment. We found that phenformin selectively inhibits G-MDSCs in vivo and in vitro. More importantly, we found that treatment with phenformin enhances the effect of anti-PD-1 immune checkpoint blockade in a genetically engineered $Braf^{V600E}/Pten^{null}$- (BRAF/PTEN-) driven mouse model of melanoma. These findings provide a rational basis for future clinical evaluation of phenformin/anti-PD-1 combination therapy.

RESULTS

We first determined whether phenformin and metformin modulate MDSC accumulation in the spleens of tumor-bearing mice. Splenocytes from immune competent FVB mice harboring BP01 mouse melanoma allografts were harvested and evaluated by flow cytometry for the proportion of G-MDSCs ($CD11b^+Ly6G^{hi}Ly6C^{int}$) and M-MDSCs ($CD11b^+Ly6C^{hi}Ly6G^{lo}$) (Figure 1a). We found a significant decrease of G-MDSCs, but not M-MDSCs, in the spleens of phenformin-treated mice compared with those treated with either metformin or the vehicle control group (Figure 1b and c). Neither phenformin nor metformin significantly changed the proportions of total macrophages, M2 macrophages, $CD4^+$ T cells, $CD8^+$ T cells, or regulatory T cells in the spleens of these mice (see Supplementary Figure S1a–e online). These results support that treatment by phenformin, but not metformin, selectively reduces the proportion of G-MDSCs in the spleens of tumor-bearing mice. We previously observed similar differential effects of phenformin and metformin on inhibiting melanoma cell proliferation, and the difference was shown to be attributable to the low expression levels of OCT2 in melanoma cells, which is required for uptake of metformin, but not the more lipophilic phenformin (Yuan et al., 2013). Similarly, we found that OCT2 expresses at a very low level in mouse MDSCs, compared with mouse liver, kidney, or several human cancer cell lines (see Supplementary Figure S2a and b online).

Next, we determined the effects of phenformin on MDSCs ex vivo. Bone marrow (BM) cells from naïve FVB mice were co-cultured with BP01 tumor cells to generate BM-derived MDSCs (BM-MDSCs). Treatment of these cells with phenformin, but not metformin, significantly decreased the proportion of G-MDSCs, but not M-MDSCs, in BM cells after 24 hours (Figure 2a and b). Similar effects were also obtained with the AMP mimetic and AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Figure 2a and b). No changes in total BM cells were observed. Co-treatment with Compound C, an AMPK inhibitor, rescued the phenformin-induced decrease of G-MDSCs (Figure 2c), suggesting that the effect of phenformin on G-MDSCs is dependent on AMPK. In addition, phenformin, but not metformin, significantly increased the proportion of Annexin V^+ cells and inhibited BrdU incorporation in the G-MDSC population (see Supplementary Figure S2c and d). These results indicate that phenformin decreases the proportion of G-MDSCs by inhibiting the proliferation and increasing apoptosis of these cells.

In contrast to M-MDSCs, G-MDSCs primarily use ROS as the main mechanism of immune suppression (Youn et al., 2008). As expected, we found that $CD11b^+Ly6G^+$ G-MDSCs derived from BM are characterized by higher endogenous ROS levels, as measured by the oxidation-sensitive fluorescent dye dichlorodihydrofluorescein diacetate, compared with $CD11b^+Ly6G^-$ cells (Figure 2d). Biguanides have been previously found to stimulate H_2O_2 production by the complex I flavin in isolated bovine heart mitochondrial fractions (Bridges et al., 2014). Indeed, we found that phenformin significantly increased dichlorodihydrofluorescein diacetate staining in G-MDSCs (Figure 2e). Co-treatment with the anti-oxidant N-acetyl-L-cysteine dampened ROS levels in these cells and rescued the effect of phenformin on reducing the numbers of G-MDSCs (Figure 2e and f), indicating that the additional increase of ROS induced by phenformin treatment may reach a toxic threshold level in G-MDSCs and contributes to its deleterious effects on these cells.

We also carried out quantitative PCR analyses on BM-MDSCs to assess the effects of phenformin on the expression of several key proteins that are important for the immune suppressive functions of MDSCs (Marvel and Gabrilovich, 2015). As shown in Supplementary Figure S3a–c, phenformin significantly decreased the expression of arginase 1, S100A8, and S100A9. Interestingly, phenformin did not cause significant changes in the expression of inducible nitric oxide synthase (see Supplementary Figure S3d), which is more important for the immune suppressive function of M-MDSCs than of G-MDSCs (Movahedi et al., 2008). Consistent with a role of phenformin in increasing ROS levels in G-MDSCs, we found that the mRNA expression of a major antioxidant enzyme, nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase 1, dropped remarkably upon treatment of phenformin (see Supplementary Figure S3e). Moreover, we found that phenformin also decreased the amount of CHOP, a stress sensor protein that is critical for the activity of MDSCs (Thevenot et al., 2014) (see Supplementary Figure S3f). These results together suggest that, in addition to reducing the number of G-MDSCs, phenformin may also impair the immune-suppressive function of MDSCs. Future studies will be carried out to further investigate the molecular mechanisms underlying the inhibitory effects of phenformin on G-MDSCs.

Therapeutic immune checkpoint blockade that targets cell surface inhibitory checkpoint regulators of T-cell activation, such as CTLA-4 and PD-1, has shown significant clinical benefits in a subset of melanoma patients (Sharma and Allison, 2015). Although most of the clinical responses to these therapies appear durable, the overall response rate remains low. Given the inhibitory effects of phenformin on immune-suppressive MDSCs, we sought to test whether phenformin may enhance the efficacy of anti-PD-1 checkpoint blockade using the BRAF/PTEN genetically engineered mouse model. We found that treatment with anti-PD1 antibody or phenformin alone only slightly inhibited tumor growth compared with IgG isotype control (Figure 3a–c). However, the combination of both phenformin and anti-PD1 antibody significantly inhibited tumor growth (Figure 3b and c). Unlike phenformin, we found that metformin had minimal effects by itself or in combination with anti-PD-1 antibody in this tumor model (see Supplementary Figure S4a and b online). Phenformin alone or in combination with anti-PD-1 significantly decreased the proportion of G-MDSCs, but not M-MDSCs, within the tumor and spleen (Figure 4a–d).