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Original Article

Differential effects of poly(ADP ribose) polymerase inhibitor-based metronomic therapy on programmed death-ligand 1 and matrix-associated factors in human myeloid cells

Salome V Ibba[1,2], Hanh H Luu[1], A Hamid Boulares[1,2]

[1]*The Stanley Scott Cancer Center/Louisiana Cancer Research Center, New Orleans, LA, USA;* [2]*Department of Interdisciplinary Oncology, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, USA*

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Abstract: We recently showed that while partial poly(ADP-ribose) polymerase (PARP)-1 inhibition with a low metronomic sub-half-maximal inhibitory concentration/dose (IC50) of olaparib provides superior protection against colon cancer in mice compared to complete inhibition by blocking the suppressive function of myeloid-derived suppressor cells (MDSCs) and synergizing with anti-program cell death (PD)-1-based immunotherapy. Here, we examined whether PARP inhibitors (PARPi) exert effects on human myeloid cells that alter T cell function (e.g. PD-ligand (L)1) or metastasis/tumor microenvironment-associated factors (e.g. tissue inhibitor of matrix metalloproteinases (MMPs) (TIMP)-2 and MMPs activity). We show that olaparib-based metronomic therapy induced a marginal increase in PD-L1 expression in MDSCs-enriched cells, decreased its expression in dendritic cells (DCs)-enriched cells, and caused little to no effect on macrophage-enriched cells. Interestingly, MDSCs-enriched cells also expressed low levels of PARP-1 while dendritic cells and macrophages expressed high levels of the protein. Bone marrow progenitors expressed no PD-L1; however, when differentiated into MDSCs, the expression was high displaying higher glycosylation levels compared to those observed in peripheral blood mononuclear cells (PBMCs)-derived cells. Contrary to reported effects on cancer cells, the sub-IC50 or moderate olaparib concentration caused substantial decrease in PD-L1. A sub-IC50 concentration of other clinically used PARPi (rucaparib, niraparib, and talazoparib) as well as the failed PARPi, iniparib, exerted similar effects. Furthermore, PARPi-based metronomic therapy reprogramed myeloid cells with the potential to stabilize intratumoral matrix by increasing secreted-TIMP-2 with a differential reduction in MMP-2/MMP-9 activity. Thus, PARPi-based metronomic therapy may promote functional changes in myeloid cells that provide an additional rationale for combining it with immunotherapy. Our results also provide new opportunities for iniparib in cancer therapy.

Keywords: PARP inhibitors, metronomic therapy, human myeloid cells, PD-L1, TIMP-2, immunotherapy

Introduction

Poly(ADP-ribose) polymerase inhibitors (PARPi) (e.g. olaparib, rucaparib, velaparib and talazoparib) remain important options for ovarian and breast cancer patients primarily those with mutations in the *breast cancer gene (brca)1/brca2* [1]. These inhibitors are also recommended as maintenance therapies for recurrent epithelial ovarian, fallopian tube, and primary peritoneal cancers [1]. Currently, PARPi are being explored in BRCA-proficient cancers upon the increasing evidence that these drugs may exhibit traits that are still related to the generation of double-strand breaks (DSBs) such as the stimulator of interferon genes (STING) pathway [2] or completely unrelated such as the promotion of programmed cell death (PD)-ligand (PD-L)1 expression in cancer cells and its connection to immunotherapy [3]. We recently reported that partial PARP-1 inhibition with low metronomic doses of PARPi or by gene heterozygosity protects against colitis- or *adenomatous polyposis coli/multiple intestinal neoplasia* (APC^{Min})-mediated intestinal tumorigenesis; while extensive inhibition achieved by

gene knockout or a high PARPi dose, is either ineffective or aggravating [4]. A strong connection was established between PARP-1 and the suppressive function of myeloid-derived suppressor cells (MDSCs). A sub-IC50 dose of the PARPi olaparib or *PARP-1*-heterozygosity was sufficient to block tumorigenesis in a syngeneic colon cancer model by modulating the suppressive function, but not intratumoral migration or differentiation, of MDSCs with concomitant increases in intratumoral T-cell function and cytotoxicity. It appears that PARP-1 regulates the expression of factors that are key to the suppressive function of MDSCs, namely: arginase-1, inducible NO synthase (iNOS), and cyclooxygenase (COX)-2. Interestingly, these effects did not involve trapping of PARP-1 onto chromatin or the STING pathway. More importantly, we found that an olaparib-based metronomic regimen was highly synergistic with anti-PD-1-based immunotherapy, which culminated in a complete eradication of microsatellite instability $(MSI)^{high}$ or a reduction of microsatellite stable (MSS) colon tumors in syngeneic mouse models.

The abovementioned study and additional studies from our laboratory and others provide substantial evidence demonstrating the roles of PARP-1 (or PARP-2) in physiological and pathological processes such as cancer, asthma, diabetes, atherosclerosis, and in molecular processes that include transcriptional events, regulation of protein function, trafficking, or integrity [5]. The primary mechanism by which PARPi exert their effects is by promoting synthetic lethality upon the consequent accumulation of fatal levels of DSBs in BRCA-deficient cancer cells [6, 7]. Trapping of PARP-1 and PARP-2 on DSBs, rather than inhibition of the activity of the enzymes, is believed to be the driving force in promoting synthetic lethality [8] although this theory was recently challenged [9]. However, the mechanism by which PARP-1 regulates immune responses may be entirely different since it entails mechanisms that do not involve DSB generation. Several studies reported a link between PARPi and PD-L1 expression; however, whether there is a strict involvement of BRCA-deficiency in this relationship remains to be to be established [1]. Evidence suggests that an increase in PD-L1 expression may enhance the success of checkpoint inhibitors, such as anti-PD-1 or anti-PD-L1, against a number of cancers [10]. The primary focus of the aforementioned studies is the expression of PD-L1 on cancer cells; an increase in the levels of PD-L1 on immune cells such as bone marrow myeloid cells may be problematic. Indeed, recently, PD-L1 expression on dendritic cells (DCs) was shown to attenuate T cell activation and to influence response to immune checkpoint blockade [11]. Accordingly, the outcome of increasing PD-L1 in immune cells, as a possible effect of PARPi, may be limiting the success of immune checkpoint inhibitors.

Another aspect of PARP inhibition that may affect function of myeloid cells and is relevant to carcinogenesis and metastasis, is the fact that it may influence the integrity of tissue matrix. Indeed, we reported that partial PARP inhibition stabilizes atherosclerotic plaques in high fat diet-fed apolipoprotein $(Apo)E^{-/-}$ mice by increasing tissue inhibitor of matrix metalloproteinases (MMPs) (TIMP)-2 with a demonstration that PARP-1 regulates TIMP-2 gene at the promoter level [12, 13]. It is noteworthy that TIMP-2 expression is highly linked to matrix stability of primary tumors and is key to protection against metastasis [14].

It is unlikely that the effects of PARPi, especially at low concentrations, on cancer cells would be similar to those on immune cells. Accordingly, this report expands on the effects of PARPi-based metronomic therapy in immune cells with a particular focus on the link between PARPi and PD-L1. It also examines whether these drugs promote functional changes in MDSCs that would make them display more anti-tumor effects by promoting factors of matrix stability such as TIMP-2.

Materials and methods

Peripheral blood mononuclear cells (PBMCs), bone marrow cells, differentiation conditions, and treatment with PARPi

PBMCs were purchased from LaCell/Obatala Sciences (New Orleans, LA). To enrich for MDSCs-like cells, PBMCs were cultured in interleukin (IL)-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) for 5 days. For DC enrichment, PBMCs were cultured in the presence of GM-CSF and IL-4 for 7 days. As for macrophage enrichment, cells were cultured in

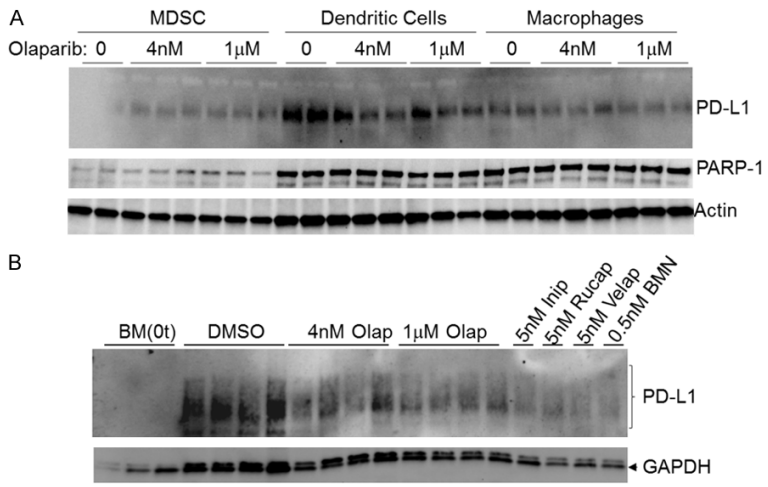

Figure 1. Differential effects of PARPi-based metronomic therapy on PD-L1 expression in differentiated hematopoietic cells derived from human PBMCs or bone marrow. A. PBMCs were cultured in specific media condition to enrich for MDSCs, DCs, or macrophages in the absence or presence of the PARPi olaparib at the indicated concentrations. Treatment was started after 24 h in differentiation media. After the respective differentiation conditions, cells were collected and protein extracts were subjected to immunoblot analysis with antibodies to PD-L1, PARP-1, or actin. B. BM-derived MDSCs were generated in the presence or absence of the indicated concentrations of PARPi. Treatment with PARPi started after 24 h of incubation in differentiation media. Protein extracts were subjected to immunoblot analysis with antibodies to PD-L1 or GAPDH. Abbreviations: Bone Marrows (BM), Peripheral Blood Mononuclear Cells (PBMCs), Myeloid-Derived Suppressor Cells (MDSCs), Programmed Death (PD), Polymerase Inhibitor (PARPi), Dendritic Cells (DCs).

media containing GM-CSF for 7 days. Bone marrows (BM) from healthy donors were purchased from Lonza. Fresh BM precursors were isolated through gradient centrifugation using a standard Ficoll gradient. The isolated cells were then cultured for 6 days in media containing a combination of human recombinant G-CSF, GM-CSF, and IL-6 at 40 ng/ml each (R&D Systems) essentially as described [4]. After 6 days, a routine percentage evaluation of MDSC was measured using a flow cytometry analysis with a total myeloid cells as cluster of differentiation (CD)33 cells were >75% of the total (polymorphonuclear leukocytes (PMN)-MDSCs: ~44%; Monocytic-MDSCs: ~30%; and early stage-MDSCs: ~5%). The suppressive function of MDSCs were assessed by a co-culture with CD3/CD28-stimulated carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells as described [4].

Immunoblot analysis and zymography

Cells were collected and processed for whole cell extraction followed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes to be probed with the indicated antibodies. The following antibodies were used in the study: PD-L1, CST #13684 (Cell Signaling); PARP-1, MA5-15031 (Invitrogen); glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) (G-9) sc-365062 (Santa Cruz Biotechnology); actin (C-2) sc-8432 (Santa Cruz Biotechnology); and TIMP-2 AF971 (R&D Biosystems). Protein expression signals were developed by enhanced chemiluminescence (Pierce, ThermoFisher Scientific) and were read by a G:Box Gel Image Analysis System (Syngene, Cambridge, UK) equipped with the GeneSys image capture software. Gelatinolytic activity in media of cultured cells was assessed by gelatin zymography using standard procedures and as described [15].

Results and discussion

Differential effects of PARPi-based metronomic therapy on PD-L1 expression in differentiated human myeloid cells

PARPi have been shown to increase PD-L1 expression [10]. PD-L1-expressing cancers are considered good candidates for checkpoint inhibitor-based immunotherapy. However, PD-L1 expression on immune cells (especially those that promote cancer progression) is problematic and may actually hamper the beneficial effects of immunotherapy [11]. To this end, we exposed PBMCs to conditions conducive to an enrichment of MDSC, DC, or macrophage populations in the absence or presence of metronomic concentrations of the PARPi, olaparib. The initial interesting difference is the low levels of PARP-1 in MDSC-enriched population compared to those observed in DCs and macrophages (**Figure 1A**). Similar results were observed when murine cells were used (data not shown). There was no apparent effect of olaparib on PARP-1 levels in the three cell popu-

lations. Although this is the first report of a difference in PARP-1 between MDSC-like cells and DCs and macrophages, it is predictable, since MDSCs as immature cells, and undifferentiated human cells such as monocytes were reported to express lower levels of PARP-1 compared to macrophages or DCs [16]. The levels of PD-L1 were relatively different in the three different cell populations with MDSCs-enriched cells expressing the lowest and DCs-enriched cells expressing the highest. Treatment with olaparib seemed to increase the levels of PD-L1 albeit in low amounts in MDSCs. Ironically, the levels of PD-L1 in DCs-enriched cells were markedly affected by olaparib treatment even at the sub-IC50 concentration. As for the macrophage-enriched population, although the PARPi treatment tended to decrease the levels of PD-L1, the effect was not as drastic as that observed in similarly treated DCs-enriched cells. These results clearly show differential effects of olaparib on PD-L1 expression in different myeloid cell populations.

Presuming that bone marrow cells would be exposed to PARPi during treatment, we wished to examine the effect of PARPi on expression of PD-L1 in bone marrow-derived MDSCs. Bone marrow cells, as expected, did not express PD-L1 (**Figure 1B**); however, upon incubation in differentiation medium, the cells expressed large amounts of the protein. It is noteworthy that PD-L1 expressed in bone marrow-derived MDSCs displayed more glycosylation as indicated by a slow migration and smear-like display of the protein on SDS-PAGE. Interestingly, treatment of these cells with olaparib at sub-IC50, or a moderate or sub-IC50 concentration of the other clinically used PARPi (e.g. rucaparib, velaparib or talazoparib (BMN)), caused a marked reduction in PD-L1 levels. It is important to mention that the concentrations used in this study do not promote PARP-1 trapping in treated cells [4] and, thus, the effect of the PARPi on PD-L1 may be independent of DSBs in a manner similar to that on ARG1 and iNOS. Interestingly, iniparib also promoted a decrease in PD-L1. Of note, iniparib showed promise in treating triple negative breast cancer in combination with standard of care [17]; however, it failed in phase III clinical trials [18]. First, it was considered as a PARPi but subsequent studies disproved such activity [19, 20]. It is unclear how iniparib reduced PD-L1 expression in MDSCs. However, it may be worth exploring its effects on MDSCs' suppressive function and its potential to synergize with immunotherapy in a manner similar to that of low doses of olaparib [4]. The major conclusion of the present studies is that PARPi may be promoting unexpected effects on immune cells. Unraveling the exact effects may facilitate and expand the therapeutic use of these drugs.

Differential effects of PARPi-based metronomic therapy on factors of matrix homeostasis in differentiated human myeloid cells

We reported that PARP-1 heterozygosity or a metronomic dose of thieno[2,3-c]isoquinolin-5(4H)-one (TIQ-A), an old generation PARPi, reduces atherogenesis in high fat diet-fed ApoE$^{-/-}$ mice [12]. Interestingly, PARP-1 inhibition was also associated with an increase in plaque stability [13], in part, through a stabilization of the matrix as a consequence of an increase in tissue inhibitor of MMPs (TIMP)-2 [13]. TIMPs, including TIMP-2, are natural inhibitors of MMPs and their expression is regarded as protective against metastasis [14]. We speculated that if PARPi can change secreted TIMP-2 levels, then the outcome would be a functional change in myeloid cells that may render their role more anti-tumorigenic. **Figure 2A** shows that among these cell populations, macrophages expressed the most TIMP-2, with MDSCs and DCs expressing negligible amounts. Treatment with either olaparib concentration of MDSCs- and DCs-enriched populations promoted a slight increase in the levels of TIMP-2. However, both concentrations of the drug promoted an increase in TIMP-2 in the macrophage-enriched population; ironically, the sub-IC50 concentration of olaparib promoted a substantial increase. **Figure 2B** shows that olaparib treatment, at either concentration, promoted a substantial increase in the level of secreted TIMP-2, which was also mirrored by treatment with sub-IC50 concentration of the other PARPi as well as iniparib.

TIMPs levels are important but only in comparison to levels of activated MMPs. Accordingly, we assessed the media for MMP activity using zymography. **Figure 2C** shows that olaparib treatment at the 4 nM did not promote an obvious change on the activity of MMP-2 or MMP-9; however, the 1 μM concentration decreased the production of MMP precursors (Pro-MMP-2 and pro-MMP-9) without a pronounced effect

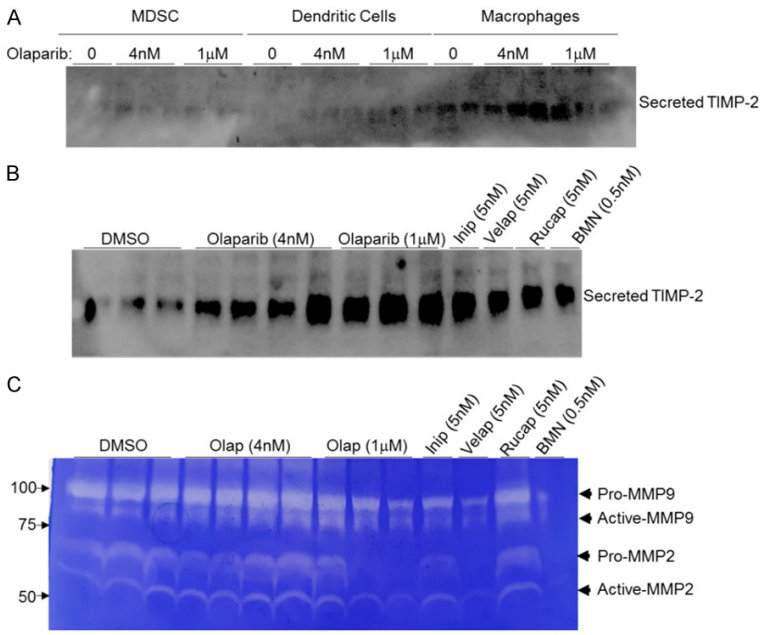

Figure 2. Differential effects of PARPi-based metronomic therapy on factors of matrix homeostasis in differentiated human hematopoietic cells. (A, B) Cell culture media from the conditions described in **Figure 1A** were concentrated four fold and equal volumes were denatured in sample buffer. Samples were then subject to immunoblot analysis with antibodies to TIMP-2. (C) Concentrated media from (B) were denatured in non-reducing and no-heating conditions prior to analysis with gelatin-containing SDS-PAGE. MMP-9/2 activity was visualized by staining gels with coomassie brilliant blue and destaining with a methanol and acetic acid mixture. Polymerase Inhibitor (PARPi).

on the active forms of the proteases. Interestingly, there were differential effects of the other PARPi on MMPs. While rucaparib did not exert any effect, velaparib appeared to affect production of the precursor proteases and their active forms. Interestingly, talazoparib almost completely eliminated the production of the precursor MMPs and the active forms. These results clearly suggest that PARPi have individual and inherent traits that may be independent of their effects on PARP-1/2 enzymatic activity.

Conclusion

Our results expand upon our recent report [4] to demonstrate that PARPi, although differentially, may be regarded as factors that change the functional properties of myeloid cells that render them anti-tumorigenic. These changes may be harnessed to provide additional benefits to their therapeutic effects and perhaps avoid potential side effects. The present results provide an opportunity to expand the utility of PARPi not only to induce synthetic lethality in BRCA-deficient cancers but also to other cancers. Such expansion may entail increasing the sensitivity of cancers to existing immunotherapies and other conditions where manipulation of the immune system may be advantageous to increase survival of cancer patients. The effects of the failed PARPi, iniparib, on PD-L1, and TIMP-2 production may resurrect the drug to be explored in enhancing the immune system to synergize with immunotherapy. Disappointingly, iniparib, to the best of our knowledge, has never been studied in the context of cancer microenvironment or immune cells. It is our hope that the present study may provide a basis for additional studies to explore the utility of iniparib in cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. A Hamid Boulares, The Stanley Scott Cancer Center/Louisiana Cancer Research Center, No. 1700 Tulane Avenue, New Orleans, LA 70112, USA. E-mail: hboulr@lsuhsc.edu

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