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Prospects of Combinatorial Approach Involving ICD Induction and Adenosine A2A Receptor Pathway Inhibition to Improve Cancer Immunotherapy

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Abstract

The purpose of this review is to discuss and summarize the prospects of combinatorial approach involving immunogenic cell death induction and immunosuppressive adenosine A2A receptor pathway inhibition in enhancing anti-tumor immunity. Majority of chemotherapeutic agents can elicit antitumor immunity and modulate the composition, density, function, and distribution of Tumor Infiltrating Lymphocytes (TILs), to influence differential therapeutic responses and prognosis in cancer patients. Accumulating evidence indicates that the clinical success of these agents not only dependents on their cytotoxic activity but also by the enhancement of pre-existing immunity. Over expression of CD39 or CD73 enzymes has been implicated in limiting the ICD caused by chemotherapeutic agents like anthracyclines and oxaliplatin. Conversion of ATP released by chemotherapeutic drugs into adenosine dampens its capacity to attract antigen presenting cells including Dendritic Cells (DC) into the proximity of dying and dead cells. In addition, released adenosine exits potent immunosuppressive activities on different immune cells through A2A receptors in the TME and contributes to the resistance against chemotherapy. Resistance either intrinsic or acquired is the major hurdle for most of the therapeutic interventions. In order to enhance immunogenic cell death by chemotherapeutic agents, it has become clear that blockade of adenosine production or its signaling need to be specifically targeted as they represent highly resistant mechanisms. Given the prominent role of adenosine mediated immune suppression and resistance to ICD induction in TME, combination strategies that involve ICD induction and adenosine signaling blockade are further warranted.

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1. Introduction

Cancer is one of the biggest and significant burdens of disease, both in terms of quality of life and morbidity. The impact of cancer is global with an estimated 19.3 million new cases and almost 10.0 million deaths worldwide[1]. Most of the standard and established therapies including chemotherapy, radiotherapy, Photo-Dynamic Therapy (PDT) have limited success due to their non-selective destructive effect on the surrounding normal cells. Immunotherapy is an innovative treatment that has become established as a fourth pillar for cancer treatment[2]. Unlike standard cancer treatments such as chemotherapy and radiotherapy, immunotherapy modulates the immune system by regulating the tumor microenvironment, so that immune cells can attack in multiple targets and directions and clear tumor cells at several important nodes[3]. However, despite the rapid advancement in the field of immune-oncology, only a subset (20-40%) of patients respond to immunotherapy[4]. In order to increase the overall response rates and efficacy of immunotherapy, many challenges need to be resolved.

Tumor Mutational Burden (TMB) is the newly established biomarker which predicts the outcome of immune checkpoint inhibitors[5]. TMB predominantly translates into a higher neoantigen load that can be expressed on the tumor cell surface, and therefore provides a higher chance of stimulating an immune reaction recognizable by a cytotoxic T-cell[6]. However, many tumors have low TMB, that can reduce antigen expression results in minimal immune stimulation[7]. Down regulation of antigen presenting cells like macrophages and dendritic cells by tumor cells contributes to immune escape, leading to scarce numbers of anti-tumor T cells in the Tumor Micro-Environment (TME) to induce tumor regression[8]. Even after the sufficient number of anti-tumor T cells infiltration, the immunosuppressive TME still restricts durable responses by utilizing multiple immunosuppressive pathways and contributes to treatment resistance[9]. To overcome these limitations, new strategies that are able to enhance infiltration of anti-tumor T cells and stimulate the dysfunctional antitumor immune system are warranted. In this review, we discussed and summarized the combinatorial approach involving immunogenic cell death induction and immunosuppressive adenosine A2A receptor pathway inhibition in enhancing anti-tumor immunity.

2. Immunogenic Cell Death: New Opportunity in Cancer Immunotherapy

The term "immunogenic Cell Death" (ICD) refers to a form of apoptosis caused by the chronic exposure or secretion of particular molecules called Damage-Associated Molecular Patterns (DAMPs) from the dying tumor cells in TME, which stimulates the dysregulated antitumor immune system by activating an adaptive immune response against Tumor Associated Antigens (TAA)[10]. DAMPs are divided according to their localization/release place and stage into three main subclasses: (1) DAMPs expressed on cell surface (e.g., HSP 70, HSP 90, and CRT,), (2) DAMPs expressed extra cellularly (e.g., Pro-inflammatory cytokines, HMGB1, and uric acid), and (3) DAMPs released as end-stage degradation factors (e.g., DNA, RNA, and ATP)[11]. DAMPs are recognized by pattern-recognition receptors including the NOD-Like Receptors (NLRs), Toll-Like Receptors (TLRs), and Retinoic acid-inducible gene-I-Like Receptors (RLRs)[12]. The key DAMPs that play a major role in ICD includes HMGB1, CRT, HSP 70 and 90, ATP, type 1 interferon and ANXA1[13]. These DAMPs, by binding to their receptors, recruit immune cells that lead to phagocytosis and trigger the activation of T lymphocytes to eliminate tumor cells[10]. Detailed discussion of the cellular and molecular mechanisms that are involved in ICD-associated DAMPs were explained in great detail previously[10,14].

2.1 ICD Inducers

Several clinically-used chemotherapeutic agents, as well as compounds of natural origins which are currently being investigated preclinically, possess immune-stimulatory function by triggering ICD.

2.2 Chemotherapeutic Agents

Conventional chemotherapeutic agents are classified generally according to their mechanisms of action: (1) DNA-intercalating agents (e.g., anthracyclines and bleomycin); (2) Alkylating agents that cross links inter- and intra-strand DNA and destabilize during replication (e.g., oxaliplatin and cyclophosphamide); (3) Antimetabolites that disrupt RNA and/or DNA synthesis (e.g., gemcitabine and 5-fluorouracil); (4) Microtubule disruptors (e.g., paclitaxel); (5) topoisomerase inhibitors

(e.g., irinotecan). Accumulating evidence indicates that the clinical success of these agents not only dependents on their cytotoxic activity but also by the enhancement of pre-existing immunity (Table 1). Based on accumulating evidence, it is now clear that some of the chemotherapeutic agents can elicit the antitumor immunity and modulate the composition, density, function, and distribution of tumor infiltrating lymphocytes (TILs), to influence differential therapeutic responses and prognosis in cancer patients[15]. These agents can induce ICD through various mechanisms to stimulate antitumor immune response at tumor microenvironment and facilitate immune cell infiltration[16].

Anthracyclines including doxorubicin, daunorubicin, and mitoxantrone are a class of widely used chemotherapeutic drugs for multiple cancers[17]. They have the potential to trigger cell death by intercalating into DNA and by suppressing DNA topoisomerase II activity[18]. Anthracyclines stimulated apoptotic cell death has also been related with potent immunogenicity involving the emission of DAMPs including HMGB1 release, CRT exposure, and ATP secretion[19]. Giglio *et al.* recently demonstrated that doxorubicin induced ICD in melanoma cells by the release of DAMPs (CRT, ATP, HMGB1)[20]. In this study, authors identified that ER stress pathway leading to activation of eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2), and eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4) as critical mediators of eukaryotic translation initiation factor 2 subunit 1 (EIF2S1) phosphorylation which in turn, regulating the CALR translocation to the outer membrane of melanoma cells[20]. Mitoxantrone induced ICD by CRT translocation involves the production of CXCL8 (or its mouse orthologue CXCL2)[21]. CXCL8/CXCL2 receptors knockdown in both human and murine cancer cells, reduced the mitoxantrone-induced CALR exposure, as well as the ability to trigger an immune response in vivo[21].

Oxaliplatin, a DNA alkylating agent developed in the 1990s, demonstrated cytotoxic effects against cancer cells, through the generation of DNA adducts and by activating pro-apoptotic transcription factors[22]. Later it was identified in colorectal cancer that oxaliplatin mediates its antitumor effects through the induction of immunogenic mode of cell death[23]. Immunogenicity of an oxaliplatin-induced cell death is governed by CRT exposure and HMGB1 release[23]. In addition to inducing ICD, oxaliplatin also decreased the expression of programmed death ligand-2 (PD-L2) leading to the inhibition of DCs and tumor cells mediated immunosuppression[23]. Oxaliplatin has also been shown to induce ICD by the secretion of CXCL10 in melanoma cells[24]. Cisplatin was previously described as unable to induce ICD due to its incapacity to promote HMGB1-dependent TLR4 stimulation and ER stress-dependent exposure of CRT on the cell surface in lung or colon cancer models[25,26]. Later it was demonstrated by multiple studies that cisplatin ability to induce CRT exposure, HMGB1 and ATP release can be depending on the concentrations used and/or cell type and/or treatment duration[26-31]. For example, cisplatin at a concentration of 20 μM with 24 h exposure did not induce ICD features[32] whereas, treatment with 2.5 μM during 24 or 48 h induced

Table 1. Conventional chemotherapeutic inducers of ICD

Class	Compounds	ICD mechanism	References
Anthracyclines	Doxorubicin & Mitoxantrone	Cell-surface CRT, ERp57, HMGB1, and ATP secretion	19-21
Alkylating agents	Oxaliplatin	Cell-surface CRT, HMGB1 and ATP secretion	24
	Cyclophosphamide	Cell-surface CRT, HMGB1 and ATP secretion	38
Antimetabolites	Gemcitabine	HMGB1 and ATP secretion	34
	5-FU	HMGB1 and ATP secretion	35

CRT exposure and ATP release, but not HMGB1 release[30]. Cisplatin in CT26 cells can induce ATP release and HMGB1, but not CRT exposure[23,31] and in EG7 cells and U2OS cells, only ATP release, but not CRT exposure[26,29]. However, in human melanoma cells, cisplatin induced CRT exposure, ATP and HMGB1 release[31].

Gemcitabine, a standard chemotherapeutic agent for various solid tumors, could trigger immunostimulatory DAMPs release (e.g., HMGB1, CRT, and HSP70) and MHC Class I expression by cancer cells[33,34]. However, gemcitabine is unable to induce ICD due to the fact that it also triggers prostaglandin E2 release as an inhibitory DAMP to counterbalance immunostimulatory DAMPs[33]. The impact of 5-fluorouracil (5-FU) on ICD, remains unclear, with controversial results about HMGB1 release[35] and calreticulin exposure in some cancer models[36,37]. Although the majority of chemotherapeutic drugs are incapable of ICD induction, their efficacy can be improved significantly by the combination of agents that produce ICD-associated DAMPs. Therefore, in order to improve chemotherapy regimens, the effects of these agents on antitumor immune response need to be considered.

Abbreviations: ICD, immunogenic cell death; CRT, calreticulin; HMGB1, high-mobility group protein B1;

Table 2. Natural compound inducers of ICD

Class	Compound	Relevant ICD mechanisms	References
Cardiac glycosides		CRT exposure, ATP secretion, HMGB1 release	59
Microtubule Destabilizing agents	Colchicine	Release of HMGB1, HSP70 and 90	44
	Epothilone	CRT exposure, increased expression of HLA Class I, and inflammatory cytokines	45
Hypericin derivatives		CRT exposure, IL6 secretion, DC maturation, CD4+/CD8+ T-cell proliferation, IFN gamma production, activation of EIF2AK3, and DDIT3, induction of BCL2L11 and PMAIP1, decrease of MCL1	49
Other compounds	Shikonin	CRT exposure, release of HSP70	51
	Septacidin	CRT exposure, HGMB1 and ATP release	54
	Wogonin	ER stress, ROS production, phosphorylation of EIF2AK3 and EIF2S1	55
	Vitamin E succinate	ROS production, activation of DCs	56
	Capsaicin	CRT exposure, HSP70 and HSP90, ATP release, phagocytosis by DCs, IFN gamma secretion	57
	Docosahexaenoic acid	CRT exposure, involvement of ROS and HSP90 HMGB1 release, activation of DCs	58

ATP, adenosine triphosphate; PDIA3 protein disulfide isomerase family A member 3 (ERp57).

2.3 Natural Agents

Though most naturally occurring compounds are primarily investigated for their anti-inflammatory and antioxidant activity, the effects of these compounds on the immune system is becoming a growing area of interest. Only a limited number of research teams screened the ability of naturally occurring anti-cancer agents to trigger the appearance of an ICD phenotype. Among the screened natural ICD inducers, only a limited number of compounds are well characterized[39]. Discovery of novel plant-derived ICD inducers that are capable of eliciting immunogenic 'eat me' signals is also crucial to supplement present chemotherapies. Interestingly, plant-derived pre-clinically investigated compounds such as cardiac glycosides or hypericin derivatives or microtubule destabilizing agents like colchicine showed promising potential as novel inducers of ICD hallmarks (Table 2).

Cardiac Glycosides (CGs) are a popular plant-derived compound with their well-known pharmacological activity against obstructive heart diseases. Due to their inhibitory activity on Na/K ATPase, they demonstrated potent anti-cancer activity by inducing necrosis or apoptosis accompanied by induction of mitophagy/autophagy in solid tumors[40]. Menger *et al.* showed that CGs including digitoxin and digoxin as well as lanatoside C and ouabain behave as potent inducers of ICD only in immunocompetent mice[41]. Moreover, a combination of chemotherapy and CGs were able to "vaccinate" syngeneic mice against injection of the same type of living cells[41]. In addition, retrospective clinical studies showed that co-administration of digoxin during chemotherapy significantly improved overall survival in colorectal, breast, hepatocellular, and head and neck carcinoma[41]. Similar observations were made with digoxin and digitoxin treatment, thus confirming that CGs induction of ICD alone or in combination with chemotherapeutic drugs[42].

2.3.1 Microtubule-Destabilizing Agents (MDAs)

Colchicine and Vinca alkaloids are two of the first discovered MDAs[43]. Colchicine and its derivatives have been shown to induce ICD with the release of DAMPs including HMGB1, HSP70, and HSP90 without affecting CRT exposure in B16F10 melanoma cells[44]. DC vaccines in combination with colchicine treated tumor cell lysates showed significant reduction in tumor growth, increased activity of cytotoxic T-lymphocyte against tumors, and increased survival rate of treated mice[44]. Epothilones derived from *myxobacterium sporangium cellulosum*[45] enhanced the recognition of ovarian cancer cells by immune cells by increasing the surface expression of MHC I and inflammatory cytokines[46]. It also triggered CRT exposure in tumor protein (TP)53-deficient murine mammary gland cells and in human cell lines[47].

2.3.2 Hypericin Derivatives

Hypericin extracted from Saint John's wort, *Hypericum perforatum* is a cytotoxic anthraquinone derivative[48]. Hypericin-based photodynamic therapy has been shown to induce ICD by CALR exposure and HSP70[49].

2.3.3 Other Compounds

Shikonin, an anticancer and anti-inflammatory phytochemical extracted from the dried root of *Lithospermum erythrorhizon* was previously used as a traditional Chinese remedy[50]. Chen *et al.* demonstrated that shikonin kills cancer cells by inducing controlled necrosis as well as mitochondrial apoptosis[51]. Also demonstrated that shikonin-induced cell death involves the release of DAMPs and DCs activation leading to enhanced priming of Th1/ Th17 effector T-cells[51]. In addition, it was observed that shikonin treatment efficiently induced ICD of 4T1 murine cells, and DC vaccines pulsed with shikonin-treated tumor cell lysates significantly suppressed Treg cell expansion in test mice and rapamycin-enhanced metastasis[52]. Septacidin is an L-heptopyranose, extracted from Streptomyces fimbriatus with anti-cancer, analgesic, and antifungal activities[53]. Septacidin has been shown to promote ATP secretion, HGMB1 release and CALR exposure from murine fibrosarcoma MCA205 and human osteosarcoma U2OS[54]. Similarly, wogonin extracted from *Scutellaria baicalensis*, vitamin E succinate, capsaicin extracted from capsicum and n3-polyunsaturated fatty acid docosahexaenoic acid have been shown to induce DAMPs in multiple murine and human cell lines[55-58].

Abbreviations: ICD, immunogenic cell death; ATP adenosine triphosphate; CRT calreticulin; HMGB1 high mobility group box 1; HSP heat shock protein; ER endoplasmic reticulum; ROS reactive oxygen species; EIF2S1 eukaryotic translation initiation factor 2 subunit alpha; EIF2AK eukaryotic translation initiation factor 2 alpha kinase; DC dendritic cells; IFN interferon; BCL2L11 BCL2 like 11 (Bim); HLA human leukocyte antigen; DDIT3 DNA damage inducible transcript 3 (CHOP); PMAIP1 phorbol-12-myristate-13-acetate-induced protein 1; IL interleukin; MCL1 myeloid cell leukemia.

3. Adenosine A2A Receptor Pathway

Wolberg *et al.* (1975)[60], first reported that adenosine as an immunosuppressive factor, and demonstrated that adenosine inhibits lymphocyte-mediated cytolysis possibly by increasing intracellular cAMP levels. The extracellular concentration of adenosine is generally low under normal physiological conditions (lower than 1 μM). However, under metabolically stressful conditions (like hypoxia) of solid tumors, the concentration of adenosine increases drastically (over 100 μM)[61,62]. It was reported that the increased concentration of adenosine in the tumor microenvironment (TME), acts as a "protective ring" to the tumors to escape from the immune system[63]. Following its extracellular release, adenosine elicits its immunosuppressive activity by engaging cell surface adenosine receptors on target cells. There are four adenosine receptors (A1, A2A, A2B, and A3), all coupled to G-proteins, and each receptor has distinct affinity for adenosine and specific distribution[64]. A1, A2A, and A3 have a high affinity to extracellular adenosine (EC50 in the range of 0.1–0.7 μM), while A2B has the lowest affinity (EC50 of 15–25 μM) suggesting that it is activated only when there is excess accumulation of extracellular adenosine, such as in the TME or under other pathological conditions[65]. Regarding the immunosuppressive actions of adenosine, A2A has been demonstrated as a dominant receptor for functional inhibition of immune cells through increased intracellular cAMP. Genetic knockout of the A2A suppressed adenosine mediated immunosuppressive activities, and small molecule A2A receptor antagonists have similar effects[66]. Adenosine A2A receptor is a high-affinity receptor and is expressed in many immune cells including T cells, macrophages, natural killer T (NKT) cells, NK cells, dendritic cells (DCs) and in tumor stromal cells[67]. Upon binding of adenosine to the A2A receptor, it triggers adenylyl cyclase activity with subsequent increase in intracellular cAMP[67]. cAMP has a profound impact on a wide range of immune cells through the activation of protein kinase A (PKA)[67]. In effector T cells, activation of A2A receptor leads to increased PKA activity which in turn inhibits the activity of T cells by multiple mechanisms, including: 1) TCR signaling inhibition by blocking Lympho-Cyte-specific protein tyrosine Kinase (LCK) dependent activation of Zeta-chain-Associated Protein kinase 70 (ZAP70)[68,69]; 2) Multiple MAP kinases (ERK1, JNK) activity suppression[69,70]; 3) CREB mediated inhibition of NF-κB and nuclear factor of activated T cells (NF-AT) signaling[71]; 4) Inhibition of PKC activity, which is critical for effector T cell activation[72]. A2A receptor signaling in CD4 T cells decreases the expression of the costimulatory receptor CD28 by reducing IL-2 secretion[73]. A2A receptor signaling in effector CD8 T cells, inhibits its cell proliferation, reduces cytokine production (IL-2, TNFα, & INFγ), and cytotoxicity[73,74]. A2Areceptor signaling also stabilizes regulatory T cells (Tregs) by increased expression of their crucial regulatory transcription factor, FOXP3[75]. Furthermore, A2A receptor signaling on both effector T cells and Tregs enhances the expression of immune checkpoint receptors, including CTLA-4, PD-1, and LAG-3[76,77]. Adenosine signaling on macrophages reduces their pro-inflammatory activity by inhibition the release of TNFα[78], IL-2[79], macrophage inflammatory protein-1α (MIP1α)[80], superoxide[81] and nitric oxide[82]. A2A receptor signaling on NK cells restricts their maturation[83] and proliferation[83] and decreases their ability to produce TNFα[84] and IFNγ[85]. In addition, A2A receptor signaling on tumor stromal cells downregulates the adhesion molecules including Vascular Cell Adhesion Molecule-1 (VCAM-1), Inter-Cellular Adhesion Molecule-1 (ICAM-1), or P-selectin leading to decreased infiltration of TILs[86].

3.1 A2A Receptor Blockade in Cancer Immunotherapy

As mentioned above, A2A is the over expressed and predominant receptor that exerts potent immunosuppressive effects on the TME. Accumulating evidence indicated the potential of targeting A2A receptors

in restoring the antitumor activity of multiple immune cell subsets. Akio *et al*. demonstrated pharmacological A2A receptor blockade has been shown to increase CD8+ T cell-mediated tumor regression in a poorly immunogenic LL-LCMV tumor model and sarcoma model[66]. In mouse models of melanoma and breast carcinoma, A2A receptor antagonists have been shown to reduce the incidence of metastasis[87]. Furthermore, combination of A2A receptor inhibition with the blockade of immune checkpoints like PD-1[88], and CTLA-4[89], and doxorubicin[90] has been reported synergistic antitumor effects. Similar antitumor effects including, slowed growth of breast and bladder tumors[90] decreased prostate cancer cell proliferation[91]. MDSCs mediated immune suppression reduction and tumor growth[92] and inhibition of VEGF production and angiogenesis[93] were observed with A2B receptor blockade. Recently, it has been shown that blockade of adenosine receptors by methyl xanthine derivative like caffeine, increased cytotoxic T cell infiltration, decreased Tregs infiltration and decreased PD-1 expression on infiltrated T lymphocytes in 3-MCA induced tumor model[94]. In addition, caffeine in combination with anti-PD1 monoclonal antibody enhanced antitumor immune response in carcinogen-induced and B16F10 melanoma tumor models[95]. Overall, multiple studies have shown the potential of A2A receptor antagonists as a promising target for cancer immunotherapy[96-98]. There are several A2A receptor antagonists or dual A2A/A2B antagonists being evaluated preclinically as well as clinically[99]. A2AR antagonists that are currently being clinically in solid tumors include, Corvus Pharmaceuticals CPI-444 (NCT02655822), AstraZeneca's AZD4635 (NCT02740985), and Novartis' NIR178 (NCT02403193, NCT03207867).

4. Therapies Combining Adenosine A2A Receptor Antagonists and Chemotherapeutic ICD Inducers

Over expression of CD39 or CD73 enzymes has been implicated in limiting the ICD caused by chemotherapeutic agents like anthracyclines and oxaliplatin[100]. One of the hallmarks of ICD is ATP secretion, which occurs in the cells that expose phosphatidylserine on their cell surface[29]. Over expression of CD39 and CD73 on tumor cells converts the chemotherapy induced ATP into adenosine[100]. Conversion of ATP released by chemotherapeutic drugs into adenosine, dampens its capacity to attract antigen presenting cells including DC into the proximity of dying and dead cells[29]. In addition, released adenosine exits potent immunosuppressive activities on different immune cells through A2A receptors in the TME and contributes to the resistance against chemotherapy. Researchers explored whether direct antagonism of A2A and A2B receptors would enhance the antitumor effects of chemotherapeutic agents. Indeed, combination of A2A[90], A2B[92,101], or dual A2A/A2B receptor antagonists[102] with oxaliplatin[92,102] doxorubicin[90,101,102], or dacarbazine[92] displayed superior tumor control[90,92,102] or longer survival[101] in tumor-bearing mice. Furthermore, mice treated with combination of PSB1115, an A2B receptor antagonist and dacarbazine, were more heavily infiltrated by NKT cells and CD8+ T cells[92]. Likewise, concomitant administration of doxorubicin or oxaliplatin along with AB928, a dual A2A and A2B antagonist, increased the intratumoral detection of CD8+ T cells[102].

5. Conclusion and Future Perspectives

Resistance either intrinsic or acquired is the major hurdle for most of the therapeutic interventions. Combinatorial approaches require a great deal of understanding and knowledge of resistance mechanisms employed by the tumor. For example, chemotherapy-induced cell death leads to the release of ATP, which in turn stimulates DC recruitment and activation. However, released ATP is catabolized to immunosuppressive adenosine by CD39/CD73, which is frequently over expressed by tumor cells. In order to enhance immunogenic cell death by chemotherapeutic agents, it has become clear that blockade of adenosine production or its signalling need to be specifically targeted as they represent highly resistant mechanisms. Hence, chemotherapy could greatly benefit from combination strategies with adenosine receptor targeting approaches to eliminate tumor cells effectively.

Combination strategies employing adenosine signaling blockade (A2A/A2B antagonism) with chemo or radio or targeted therapies are currently being

underway in the clinical stage. Given the prominent role of adenosine mediated immunosuppression and resistance to ICD induction in TME, combination strategies that involve ICD induction and adenosine signaling blockade are further warranted.

6. Conflict of Interest

None

7. Acknowledgement

None

8. References

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