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Review

Intracellular mechanisms of tumor cells' immunoresistance

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One of the main mechanisms for avoiding immune response by cancer cells is mediated by inducing an immunosuppressive environment in the tumor following activation of immune checkpoints, i.e. PD-1 or CTLA-4 receptor inhibitors on T lymphocytes. Interaction inhibition between PD-1 or CTLA-4 and their ligands (PD-L1, CD80, and CD85) leads to unblocking of the T-lymphocyte function, and thus destroys cancer cells. Certain intracellular signaling pathways are also involved in the development of tumor cell immunoresistance. Immunosuppressive pathways' activation blocking may increase the immunological anti-tumor control.

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Abbreviations: Akt, protein kinase B; Bcl-xL, B-cell lymphoma-extra large transmembrane molecule; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; CY-202, seliciclib; dMMR, mismatch repair-deficient; IL-8, interleukin-8; LAG-3, lymphocyte activation gene 3; mCRC-metastatic colorectal cancer; MEK, activation of mitogen-activated protein kinase kinase; mTORC2, rapamycin-insensitive protein complex; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PD-L1, programmed death-ligand; PI3K, phosphoinositide 3-kinase; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase gene; TCR, T-cell receptor; TIM-3, mucin-domain-containing molecule-3; TIL, tumor-infiltrating lymphocytes; TME, tumor microenvironment; TNF-α, tumor necrosis factor α; Treg-regulatory T cells; STAT3, activator of signal transducer and activator of transcription 3

INTRODUCTION

Over 8 million people die from cancer each year. According to epidemiological estimates, in the upcoming two decades, the number of deaths will increase by 2-fold among men, and by about 1/3 in the female population. This means that the scale of morbidity and, unfortunately, mortality due to colorectal carcinoma is continously on the rise. Undoubtedly, a significant contribution to the progression of this disease is the progress of civilization and the development of the world in which we live. Patients with colon cancer are a relatively heterogeneous group and thus it is difficult to design a single effective treatment pattern. A basic element of therapy is surgical treatment, which is aimed at obtaining intestinal tissue free of cancer. Chemotherapy complements surgical intervention and is mainly based on 5-fluorouracil/folic acid, capecitabine, oxaliplatin, or irinotecan. More and more often, classical chemotherapy is supplemented with a targeted biological therapy, involving administration of antibodies directed against specific molecular structures that stimulate cancer development. This therapy is intended for strictly selected (potentially sensitive) groups of patients and includes such drugs as bevacizumab, cetuximab, panitumumab, or regorafenib (https://www.nccn.org/). The continuing growth rate of colorectal carcinoma incidence indicates a necessity for intensified research, and forces to seek novel methods for early detection and more ideal treatment for patients suffering from this type of cancer. Therapy that is currently employed is undeniably unsatisfactory.

Recently discovered phenomenon of cancer cells avoiding the immune response has become the basis for the development of a new, groundbreaking direction in cancer treatment, i.e. immunooncology. The American Society for Clinical Oncology (ASCO) in their "Clinical Cancer Advances 2016" report, published in the Journal of Clinical Oncology, deemed immunotherapy to be the greatest advance in 2016. This breakthrough discovery was further acclaimed by awarding the 2018 Nobel Prize to James P Allison and Tasuku Honjo. Restoring proper immune system function has proven to be an effective strategy for fighting cancer. Thanks to the presence of antigens on the cancer cell surface, the immune system is able to recognize it and destroy it. Activation of the immune system cells occurs according to a two signal model. Interaction of the TCR (T-cell receptor) with the MHC molecule leads to antigen presentation and activation of specific T lymphocytes that achieve full effector functions only after ligand binding to the co-stimulatory molecules of lymphocytes. Lack of the second signal results in lymphocyte anergy and a deacrese in the immune system response. Apart from activating co-stimulatory molecules, there are also molecules with immunosuppressive activity, called the immune checkpoints, which include: PD-L1, CTLA-4, LAG-3, or TIM-3 (Lynch *et al.*, 2016). The receptor pathway of programmed cell death 1 (PD-1) and its ligand PD-L1 are one of the most-studied immune checkpoints. PD-L1 is a transmembrane glycoprotein that is responsible for maintaining peripheral tolerance by limiting the T-lymphocyte activity, proliferation and effector functions. Results of large clinical trials clearly indicate that high PD-L1 expression directly correlates with cancer stage, metastasis and worse prognosis (Zhuan-Sun *et al.*, 2016, Zhang *et al.*, 2017; Wang *et al.,* 2017). This protein is a valuable prognostic biomarker and a reliable indicator of treatment effectiveness for some types of cancer (Hamanishi *et al.,* 2007; Xiang *et al.,* 2017; Zhang *et al.,* 2017).

PD-1/PD-L1 SIGNALING PATHWAY – A LINK INHIBITING THE ANTI-TUMOR IMMUNE RESPONSE

PD-1/PD-L1 signaling pathway is essential under physiological conditions, where it is responsible for

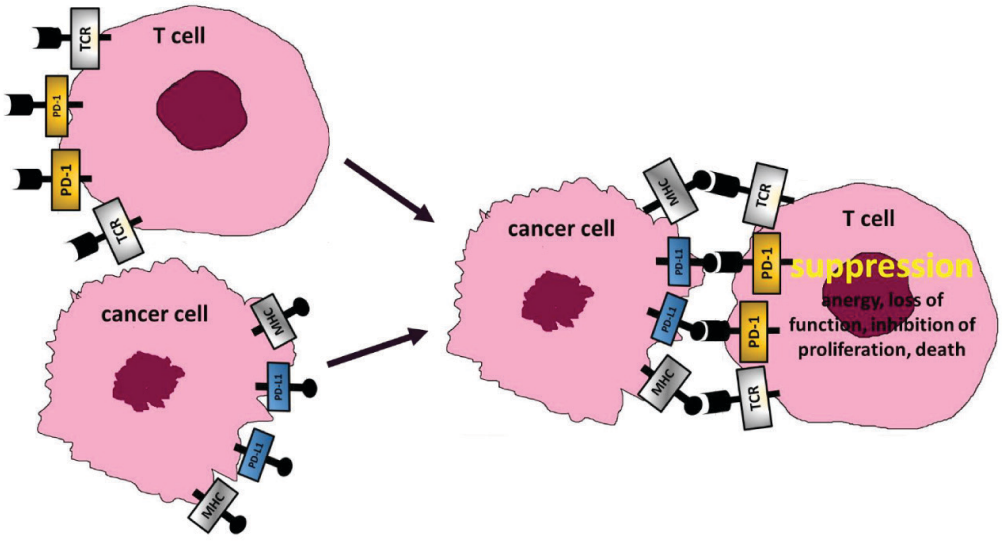

Figure 1. The PD-1/PD-L pathway.
MHC, major histocompatibility complex; PD-1, programmed death receptor 1; PD-L1, programmed death-ligand; TCR, T-cell receptor.

maintaining tolerance to its own antigens and prevents autoimmune disease development, while it also plays a key role in cancer cell "escape" from the immune surveillance (Fig. 1) (Nishimura *et al.,* 2001, Boussiotis, 2016, Valentini *et al.,* 2018). First described by Ishida and coworkers (Ishida *et al.,* 1992) the PD-1 receptor is a glycoprotein receptor composed of 288 amino acids, encoded by the PDCD-1 gene located on chromosome 2 (Ishida *et al.,* 1992; Zhang *et al.,* 2004; Wlasiuk *et al.,* 2016). It is continuously expressed, predominantly on activated T lymphocytes and macrophages, while the presence of this receptor on B lymphocyte and NK cell surfaces requires induction (Valentini *et al.,* 2018). NF-ATc1, nuclear factor c1 of activated T lymphocytes, plays an important role in regulation of PD-1 expression. Its blocking reduces PD-1 expression while a mutation in the coding gene results in a complete lack of PD-1 expression (Oestreich *et al.,* 2008). The primary role of the PD-1 receptor is to inhibit the T lymphocyte function, which is observed after receptor binding to one of the ligands on APC cells. This leads to slower cell metabolism, and thus lymphocyte depletion in their effector functions. Production of TNF-γ, TNF-α, and IL-2 is inhibited, and the amount of the anti-apoptotic Bcl-xL protein decreases, promoting the apoptosis process. While the CTLA-4 receptors inhibit T cell activation, mainly on the basis of competing for molecule CD80/CD86, the PD-1 receptor and its ligands constitute a separate signaling pathway (Riella *et al.,* 2012). Two ligands for the PD-1 receptor, PD-L1 (B7-H1, CD274), programmed death molecule 1, and PD-L2 (B7-DC, CD273), programmed death molecule 2, were identified. They are transmembrane type I glycoproteins with IgV- and IgC-like domains. PD-L1 is expressed on the surface of the T and B lymphocytes, macrophages, dendritic cells, and numerous non-hematopoietic cells, whereas PD-L2 expression is more limited (Grzywnowicz & Giannopoulos, 2012). PD-L1 expression was also detected on the cell surfaces of numerous types of cancer, including bladder, kidney, ovarian, lung, and melanoma, which was associated with poorer immune response and worse prognosis for patients. The large amounts of PD-L1 help some cancer cells to hide from an immune attack (Feld *et al.,* 2016; Lee *et al.,* 2016; Maleki *et al.,* 2017; Xiang *et al.,* 2017; Rom-Jurek *et al.,* 2018). The presence of this protein has been also demonstrated in colorectal carcinoma cells. Valentini et al. confirmed its expression on both, the tumor cells and tumor-infiltrating lymphocytes (TIL) (Valentini *et al.,* 2018). Although preliminary trials did not suggest a significant role of immunotherapy in the treatment of colorectal carcinoma (CRC), Le and coworkers (Le *et al.,* 2015) showed a marked increase in an objective immune response rate and progression-free survival rate in patients treated with pembrolizumab. Results published in 2017 and 2018 established the PD-1 inhibitors, pembrolizumab and nivolumab, and the combination of nivolumab with the CTLA-4 inhibitor ipilimumab, as effective options for mismatch repair–deficient (dMMR) metastatic colorectal cancer (mCRC) (Le *et al.,* 2015; Smith *et al.,* 2018). Immunotherapeutic agents have grown in popularity for treating mismatch repair–deficient metastatic colorectal cancer (mCRC), becoming the standard of care in the second line. In turn, preliminary analyses of early stages of current research in patients with metastatic colorectal cancer demonstrated promising atezolizumab activity (anti-PD-L1 antibody) when used together with chemotherapy and/or targeted therapy with cobimetinib, the MEK inhibitor (Tapia *et al.,* 2018). Despite initial enthusiasm, the IMblaze370 trial failed to improve survival over standard third-line therapy for patients with chemorefractory metastatic colorectal cancer and microsatellite-stable disease (Eng *et al.,* 2019). However, recent results from phase II of the CCTG CO.26 study presented at the 2019 American Society of Clinical Oncology Annual Meeting clearly showed that the combination of the anti–PD-L1 antibody, durvalumab, and the anti–CTLA-4 antibody, tremelimumab, extended the median overall survival (OS) by 2.5 months when compared with the best supportive care alone in patients with microsatellite stable, refractory advanced CRC (6.6 *vs* 4.1 months; HR, 0.72; 95% CI, 0.54–0.97; P=0.07) (Chen *et al.,* 2019).

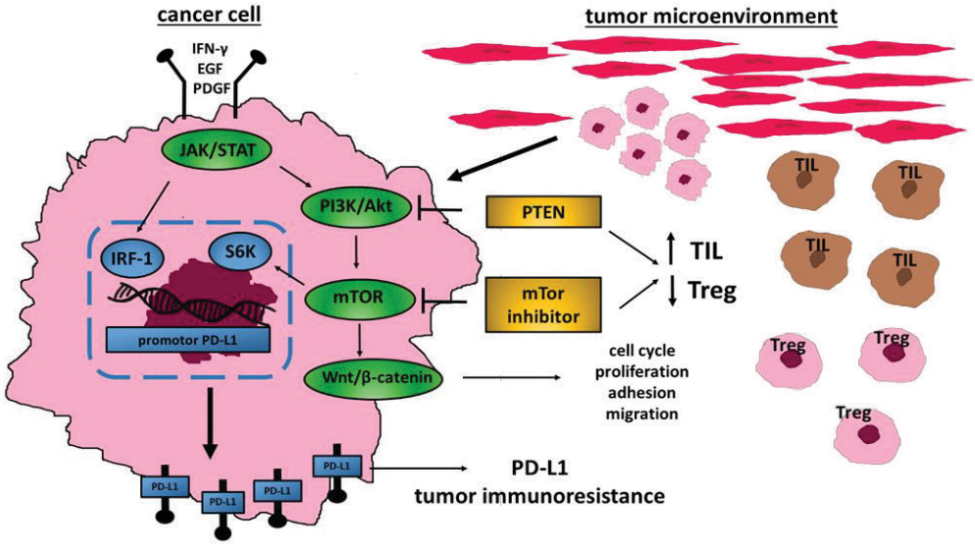

Figure 2. Selected intracellular mechanisms of tumor cell immunoresistance.
Akt, protein kinase B; IRF-1, interferon regulatory factor 6; JAK, Janus kinase; mTOR, mammalian target of rapamycin kinase; PD-L1, programmed death-ligand; PI3K, phosphoinositide 3-kinase; S6K, ribosomal protein S6 kinase; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase gene; STAT, activator of transcription protein; TIL, tumor-infiltrating lymphocytes; Treg, regulatory T cell; WNT/b, catenin signal transduction pathways made up of proteins that pass signals into a cell through cell surface receptors.

The immune checkpoint blockade therapy has achieved a remarkable success in the treatment of patients with various cancer types and is a promising novel tool in oncology. Yet, only a limited fraction of patients derive a clinical gain. The results of large clinical trials show how, despite the exceptional survival benefit obtained with immune checkpoint blockade, some populations of patients (40–60%) will not avail from this therapy (Maleki *et al.,* 2017; Catalano *et al.,* 2019). Unfortunately, factors that influence the therapy response remain not fully known. Detection of PD-L1 on the surface of tumor cells is an important and so far the most common clinically detected biomarker for predicting patient response to the anti-PD-1/PD-L1 therapy (Herbst *et al.,* 2014; Topalian *et al.*, 2014; Ansell *et al.,* 2015; Le *et al.,* 2015; Maleki *et al.,* 2017). There are also some interesting data indicatung the tumor microenvironment, the indole 2,3-dioxygenase, and finally mutational landscape, as biomarkers for predicting the response to immunotherapy (Ji *et al.,* 2012; Holmgaard *et al.,* 2013; Anagnostou *et al.,* 2017; Maleki *et al.,* 2017). It has been demonstrated that tumors with many somatic mutations due to mismatch-repair defects are more susceptible to the immune checkpoint blockade than tumors lacking this property (Le *et al.,* 2015; Deppert & Bruns, 2016). Thus, immunogenicity of the tumor antigen T-cell epitopes may also be significant for treatment success or failure. Taking into account the dynamic nature of the immune system and the multiple factors involved in the complex antitumor immune response, developing biomarkers for immunotherapeutics can be quite a challenge.

It is worth noting that using immunotherapy is costly and might have some serious adverse events associated with excessive immune activation, termed immune-related adverse events (Bajwa *et al.,* 2019). This upregulation can potentially affect one or more organs, leading to pneumonitis, hypothyroidism, hepatitis, colitis, as well as more general adverse events, such as fatigue, diarrhea, and rash (Ai & Curran, 2015; Turnis *et al.,* 2015; Bajwa *et al.,* 2019; Da1 *et al.,* 2020). Many events can be life-threatening, which requires discontinuation of treatment or long-term corticosteroids (Bertrand *et al.,* 2015).

INTRACELLULAR MECHANISMS OF TUMOR CELL IMMUNE-RESISTANCE

The PI3K/Akt pathway plays a key role in regulation of processes related to cell growth, metabolism, survival and proliferation. Akt, a serine/threonine protein kinase, also called protein kinase B, is the main signal transducer in this pathway. Increased expression of this kinase is observed in numerous cancers, including breast, lung, and prostate cancer (Lastwika *et al.,* 2016). Its main cause is a mutation in the genes encoding Akt isoforms or amplification and activating gene mutation of the catalytic subunit of PI3K (the PIK3CA mutation). Increased Akt kinase activity is not usually a sufficient factor responsible for initiating the oncogenesis process, but it contributes to tumor progression by inhibiting apoptosis, promoting proliferation, migration and invasion. Moreover, recent studies indicate that the PI3K/Akt/mTOR pathway is also involved in the development of tumor cell immune-resistance. Lastwikaand coworkers (Lastwika *et al.,* 2016) showed that the m embrane PD-L1 expression in human lung cancer cells is significantly associated with mTOR kinase activation. It was proven that oncogenic activation of the Akt/mTOR pathway promotes the immune escape through enhanced PD-L1 expression (Fig. 2). Still, use of mTOR inhibitor in combination with the PD-1 antibody in the mouse lung cancer model is the cause of a significant inhibition of tumor growth, increase in TIL number, and a significant reduction in Treg number. These results indicate a significant contribution of the PI3K/Akt/mTOR pathway not only in the regulation of PD-L1 expression, but also in the development of an immunosuppressive tumor microenvironment (TME) (Lastwika *et al.,* 2016). The data in the literature suggest the existence of two mechanisms responsible for the induction of PD-L1 expression in tumor cells. The first one is related to the reaction to

changes occurring in the tumor microenvironment. Increased PD-L1 expression is a response to TIL lymphocyte attack, the presence of which indicates immunogenicity of a given tumor, and whose efficacy is significantly reduced in the presence of PD-L1 (Parsa *et al.,* 2007). Tumor-infiltrating lymphocytes (TIL) are a key component of the TME, reflecting the host antitumor immune response, defined as lymphocytes that surround, attack and eradicate tumor cells (Horton & Gajewski, 2018). TILs are thought to be suppressed by multiple immune inhibitory molecules in the tumor microenvironment and this suppression has been associated with tumor progression. Sobral-Leite et al. showed a clear association between TILs and PIK3CA mutations. Assessment of TIL subsets in terms of PI3K changes indicated that tumors with a PIK3CA mutation often possess more CD8+ cells (subset of TILs) (Sobral-Leite *et al.,* 2019). Enhancement of CD8+T cell infiltration within the tumor tissue has been also observed after treatment with PI3K pathway inhibitors (selective PI3Kβ (p110β), isoform inhibitor GSK2636771, pan-PI3K inhibitor LY29002). Increase in the number of CD8+T cell caused tumor burden and significant survival benefits in various animal models of cancer (O'Donnell *et al.,* 2017). Reduction in the CD8+ numbers and high numbers of Treg in the tumor infiltrate are associated with a poor prognosis (Zeng, 2017). Tregs represent a unique CD4+ T-cell subpopulation that induces immunological self tolerance and are in charge of suppressing activation and proliferation of autoreactive lymphocytes. Recently, they have been shown to migrate into tumors and suppress an effective anticancer response in the tumor microenvironment (Beyer & Schultze, 2009; Onishi *et al*., 2014). Their depletion is effective in enhancing immune responses. Abu-Eid et al. showed that PI3K–Akt inhibitors caused a significant antitumor effect which was Treg dependent. They inhibited activation and proliferation of Tregs with a minimal effect on conventional T cells. This effect has been observed both in vitro (human and murine CD4 T cells) and in vivo, in naive and tumor-bearing mice (Abu-Eid *et al.,* 2014).

The second mechanism responsible forinduction of PD-L1 expression, independent of the immune system activity, assumes a PTEN tumor suppressor gene mutation. This leads to loss of ability to inhibit the PI3K/Akt/mTOR/S6K pathway, which consequently results in an increased PD-L1 expression (Fig. 2; Parsa *et al.,* 2007). The phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase gene (PTEN) often mutates in the case of glioblastoma, melanoma, gastric, ovarian, kidney, breast, and lung cancers (Crowell *et al.,* 2007). Moreover, activation of mTORC2 (rapamycin-insensitive protein complex) interferes with PTEN-loss Treg stability and their ability to differentiate (Huynh *et al*., 2015). De la Iglesia and others (De la Iglesia *et al.,* 2008) demonstrated that the PTEN-loss glioblastoma do not show transcriptional repression of the interleukin-8 gene due to lack of binding of transcription factors (activator of signal transducer and activator of transcription 3-STAT3) to the IL-8 promoter. This resulted in increased transcription and expression of IL-8, which promotes glioblastoma cell proliferation and invasiveness only in a genetic PTEN-loss context (De la Iglesia *et al.,* 2008). This relationship between PTEN-loss and a selective upregulation of IL-8 signaling has been also demonstrated in prostate carcinoma (Maxwell *et al.,* 2013). Over-activation of the PI3K-AKT-mTOR pathway directly affects TME through upregulation of IL-8 (Conciatori *et al.,* 2018). Furthermore, elevations of serum IL-8 concentrations reflect tumour growth dynamics and have been considered as biomarkers for predicting response to the anti-PD-1 therapy in non-small cell lung cancer and melanoma. The most recent data from clinical and preclinical studies indicate that PI3K/Akt/mTOR inhibition may have a double benefit. On one hand, it may limit tumor growth by inhibiting cell proliferation, migration, and survival. On another hand, it may increase the immunological anti-tumor control by blocking activation of the immunosuppressive pathways and strengthening the internal immunity mechanisms (Xue *et al.,* 2015).

Another pathway involved in the phenomenon of immunosuppression, or cancer cell "escape" from the immune system surveillance, is the intracellular JAK/STAT. JAK and STAT (mainly STAT3 and STAT5) proteins control many processes associated with tumor development, including cell division and viability, new blood vessel formation, as well as immunoresistance. Increased activity of these proteins is observed in many cancers. Marzec et al. showed that the amplifiable 9p24 region contains the JAK2 gene, as well as the gene encoding PD-L1, which contributes to the development of Hodgkin's lymphoma due to weakening of the anti-tumor immune response (Marzec *et al.,* 2008). Moreover, in patients with colorectal cancer and melanoma, who are carriers of the inactivating JAK1/2 gene mutation, a complete lack of PD-L1 protein expression is observed, resulting in resistance to therapy with inhibitors of immune checkpoints (anti-PD-1) (Shin *et al.,* 2017). Another important factor regulating PD-L1 expression is the STAT3 protein. It was shown that in patients with lymphoma, characterized by NPM-ALK presence, increased activity of this protein leads to the PD-L1 over-expression (Green *et al.,* 2010; Lastwika *et al.,* 2016). NPM-ALK fusion was also shown to have the ability to activate STAT3, which is an indispensable factor for stimulating gene transcription of PD-L1 (Fig. 2; Davis *et al.,* 2010). It is worth emphasizing that the STAT3 protein negatively regulates the immune response. It inhibits the Th1 activity by increasing expression of certain immunosuppressive factors, including IL-10 (Williams *et al.,* 2004). It was also noticed that myeloid cells displaying the active form of the STAT3 protein inhibit the anti-tumor immune response (Yu *et al.,* 2007). Research conducted by Wang et al. showed that blocking the STAT3 protein in B16 murine melanoma tumors results in a tumor mass reduction and increased T lymphocyte infiltration (Wang *et al.,* 2004).

Looking for new therapeutic strategies combining anti-neoplastic activity with activation of the anti-tumor immune response is an especially urgent task for researchers, doctors, and the pharmaceutical industry. Growing knowledge in the field of molecular biology and cancer genetics has contributed to the discovery of new, effective therapeutic methods based on the intracellular signal transmission. Introduction of monoclonal antibodies and tyrosine kinase inhibitors for wide use was undoubtedly a huge success and a significant step in the fight against cancer. The special role of proteins that are overexpressed as a result of gene mutation or amplification in the course of the neoplastic process was recognized. High hopes are associated with micromolecule drugs with the ability to inhibit EGFR, VEGFR, intracellular signaling pathways, such as Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, as well as cyclin-dependent kinase inhibitors, such as roscovitine, for example. Roscovitine, 2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9H-purin-2-yl]amino]-1-butanol also called CY-202 or seliciclib, is a low molecular weight purine derivative with a charac-

teristic ring structure. It belongs to the cyclin-dependent kinase (CDK) protein inhibitors that play a key role in regulating the cell cycle, promoting its progression or transition between the individual phases. Increased CDK expression is observed in tumor cells, which may be the cause of cell cycle deregulation. CDK inhibitors inhibit cell division and induce programmed cell death. Roscovitine has the ability to inhibit numerous cyclin-dependent kinases, including: CDK1, CDK2, CDK5, CDK7, and CDK9, but also other kinases, such as CaMK2, CK1α, CK1δ, DYRK1A, EPHB2, ERK1, ERK2, FAK, and IRAK4. This compound also blocks the RNA II polymerase, lowers expression of Bcl-2, Mcl-1, and XIAP genes, and increases p53 expression, which in turn leads to cell death via apoptosis. The results of the latest research indicate that by inhibiting Cdk5 and blocking p53 degradation, roscovitine significantly weakens PD-L1 expression, promoting the anti-tumor immune response (Cortez *et al.,* 2015; Dorand *et al.,* 2016) .

Despite considerable progress in treating cancer in recent years, designing an efficient anti-neoplastic therapy is still a serious challenge for contemporary oncology. Lack of sufficient knowledge on the tumorigenesis process, the mechanisms of drug resistance, or finally the escape of cancer cells from the immune system is the cause of the uneven and difficult fight against cancer.

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