Am J Cancer Res 2021;11(11):5625-5643
www.ajcr.us /ISSN:2156-6976/ajcr0136757

Review Article

Dual anticancer role of metformin: an old drug regulating AMPK dependent/independent pathways in metabolic, oncogenic/tumorsuppresing and immunity context

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Received June 25, 2021; Accepted October 29, 2021; Epub November 15, 2021; Published November 30, 2021

Abstract: Metformin has been known to treat type 2 diabetes for decades and is widely prescribed antidiabetic drug. Recently, its anticancer potential has also been discovered. Moreover, metformin has low cost thus it has attained profound research interest. Comprehensing the complexity of the molecular regulatory networks in cancer provides a mode for advancement of research in cancer development and treatment. Metformin targets many pathways that play an important role in cancer cell survival outcome. Here, we described anticancer activity of metformin on the AMPK dependent/independent mechanisms regulating metabolism, oncogene/tumor suppressor signaling pathways together with the issue of clinical studies. We also provided brief overwiev about recently described metformin's role in cancer immunity. Insight in these complex molecular networks, will simplify application of metformin in clinical trials and contribute to improvement of anti-cancer therapy.

Keywords: Metformin, cancer, AMPK, metabolism, oncogenes, tumor suppressors, immunity

Introduction

French lilac (Galega officinalis), is a herb known for considerable time period (centuries) to decrease the symptoms of diabetes mellitus and the biguanide metformin is derived from this plant [1]. Metformin (N', N'-dimethylbiguanide), has the greatest efficacy and it is the harmless commonly recommended antidiabetic (in comparison with other biguanides) remedy for type 2 diabetes mellitus (T2DM) [2]. At this time it is concluded that metformin functions by preventing glucose production in the liver [2] and the anti-hyperglycemic action of metformin have been ascribed, in part, to elevated hepatic insulin sensitivity and enlarged glucose uptake into peripheral tissues, such as skeletal muscle and adipose tissues [3].

Metformin has a vast different medical assets. It has successfully been accepted in the administration in polycystic ovarian syndrome (PCOS) and metabolic syndrome [4]. Also metformin may be useful in the treatment of cancer, cardiovascular disease, delay in the aging process and regulation of microbiota [5]. This review will provide an overview of the recent known metabolic effects, influence on oncogenes/tumor suppresors and immunity from AMP-activated protein kinase AMPK-dependent and independent aspect in cancer cells, as well as recent clinical studies examining the ability of metformin in the cancer treatment.

Metformin in the cell

Metformin is a hydrophilic molecule and therefore cannot pass through the cell's membrane by passive diffusion. The organic cation transporter family (OCT), and specifically the organic cation transporter 1 (OCT1), is responsible for the uptake of metformin into hepatocytes and tumor cells [4]. In 2014 three groups, reported unique and convincing confirmation on the pre-

venting complex I properties by metformin implementing distinct experimental procedure [1]. The suppression of mitochondrial respiration and ATP synthesis by metformin drives to metabolic adaptations striving to retrieve cellular ATP levels [6]. This altered energy status in the cell is detected by a central energy sensor-AMP activated protein kinase (AMPK). AMPK is activated by binding of ADP or AMP molecules to a specific site on its regulatory gamma subunit [7]. Once activated, AMPK stimulates catabolic processes in which ATP is synthesized and inhibits anabolic processes where ATP is consumed and thus the cell adaptes to a state of reduced energy [7, 8]. Cancer cells that are not capable to recover from this reduced energy status may encounter apoptosis [9], and the induction of mitochondria-mediated apoptosis by metformin was described in glioma cells in 2007 [1, 10]. It is still not defined, whether AMPK plays the role of a major mediator in the action of metformin in cancer cells, therefore the effects of metformin on AMPK-dependent and AMPK-independent mechanisms in cancer cells will be reported in this review.

Metformin in cancer cells, its mode of action remains controversial

Despite extensive research about the molecular events in cancer cells, cancer remains predominant reason for life quality impairment of cancer patients and mortality in the world. Increased enthusiasm in the therapeutic ability of different antidiabetic drugs has appeared after attention that type II diabetes mellitus is linked with increased cancer risk [1].

Further research was prompted after 2 epidemiological reports suggested a link between metformin treatment and a reduced cancer risk and decreased cancer mortality in diabetic patients [11, 12]. However, it is important to note that in vitro and in vivo studies tend to overestimate the importance of metformin in cancer therapy, while meta-analyzes of randomized controlled trials do not appear to show a significant effect of metformin on cancer outcome [3].

AMPK-dependent effect of metformin on cancer cells metabolism

Among the first evidences of the AMPK importance in the antiproliferative effect of metformin are studies on ovarian and breast cancer in vitro. Since then, numerous studies have been completed scrutinizing the role of AMPK in the anticancer effect of metformin [13-15].

Although there are still ambiguities in the AMPK activation, direct and indirect mechanisms of AMPK activation can be distinguished [5].

(1) Direct activation of AMPK involves binding AMP/ADP to the gamma regulatory subunit, inducing conformational change and unmasking Thr 172 residue on the catalytic alpha subunit [5].

(2) Indirect activation of AMPK ensues via upstream kinases such as serine threonine kinase-11/LKB1, calmodulin-dependent kinase kinase 2/CAMKK2 and TGF beta-activated kinase-1/TAK [7].

It is thought that activation of the LKB1/AMPK signaling pathway may significantly contribute to the anti-cancer effect of metformin. LKB1, an upstream activator of AMPK, is a known tumor suppressor and mutations in this gene have been associated with Peutz-Jeghers syndrome, an inherited predisposing disorder characterized with development of different cancer types [16].

AMPK-dependent metformin's role in cancer cells protein metabolism

Mechanistic target of rapamycin mTOR integrates protein synthesis with growth and proliferation of cells depending on availability of nutrients. mTOR is inhibited by AMPK downstream targets Raptor and Tuberous Sclerosis Complex/TSC2 phosphorylation [17]. Subsequent to metformin activation of AMPK it can be assumed decline in pathways regulated by mTOR and prevention of cancer cell proliferation and growth (**Figure 1**). Several studies confirmed this hypothesis. In human AGS gastric adenocarcinoma cells metformin triggered intrinsic apoptotic response by AMPK stimulation and AKT/mTOR signaling disruption [18]. Another study on human gastric cancer cells reported that AMPK/mTOR pathway dependent inhibition of survivin partly contributes to metformin-induced apoptosis [19]. Study on esophageal squamous cell carcinoma in vivo validated that metformin prevented esophageal carcinogenesis by AMPK/mTOR signaling pathway

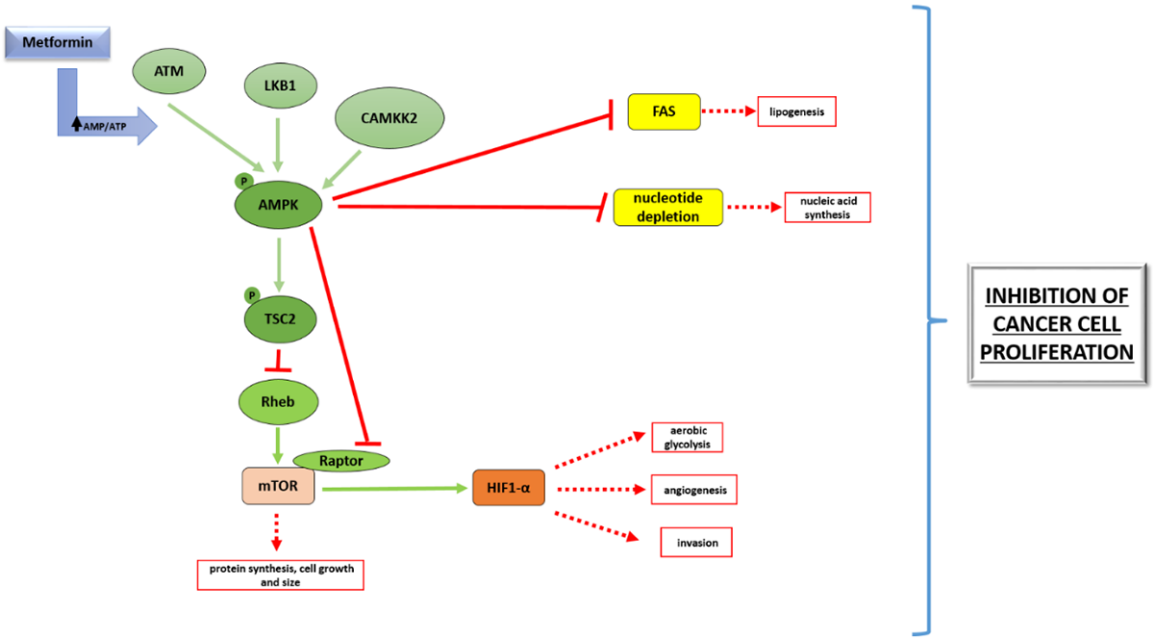

Figure 1. AMPK-dependent effect of metformin on cancer cell metabolism. Key metabolic molecules and their regulation described in review are depicted on **Figure 1**. Green arrows represent activation, while red lines show inhibitory interaction. Red squared boxes illustrate inhibition of distinct metabolic process.

[20]. Finally there is a remarkably study in breast cancer cells assembling [21] cation-selective transporter expression, the AMPK/mTOR signaling cascade and the antiproliferative role of metformin.

Apart from metabolic modulations and apoptosis regulation, it is widely accepted that AMPK activation and subsequent mTOR inhibition induces autophagy [22]. Autophagy is highly conserved evolutionary process serving for removement of old proteins and organelles in the cell. Induction of autophagy in cancer cells can have different consequences: it inhibits (cytotoxic autophagy) or promotes (cytoprotective autophagy) cancer cell survival. Metformin in myeloma cell lines induced autophagy and G0/G1 cell cycle arrest by targeting the AMPK/mTOR pathway [23]. It was also shown on melanoma cell line partially AMPK-dependent cytotoxic autophagy activation by metformin [24]. In human hepatocellular carcinoma cells metformin has induced autophagy via the AMPK-mTOR activation, but in this study the role of autophagy (cytotoxic or cytoprotective) was not clearly shown [25]. Contrarily, Chen's study on NSCLC cell lines implicated that metformin inhibits autophagy by AMPK activation and has sensitized non-small-cell-lung-carcinoma/NSCLC cells to osimertinib. Authors presumed that sustained AMPK activation induced by metformin could impede autophagy in a time-dependent manner. Ben Sahra depicted in prostate cancer cells that combination of 2-deoxy glucose and metformin has supressed autophagy and activated AMPK-dependent apoptosis, recognizing ambiguous role of AMPK modulation in cellular signaling [26].

There are also studies not precisely investigating the role of AMPK-mTOR signaling pathway in autophagy induced by metformin. It was revealed that metformin precluded cell tumorigenesis of NIH/3T3 (mouse embrionic fibroblasts cell line) via autophagy-related cell death. Authors did not analyze the role of AMPK-mTOR signaling pathway, instead showing unfolded protein response-mitogen activated protein kinases/UPR-MAPKs dependent autophagy induction by metformin. These findings proposed that autophagic cell death could be recognized as a new mechanism of disposing damaged cells serving as an attractive strategy for discarding potentialy tumorigenic cells [27]. Different than in Chen, study on NSCLC cells demonstrated that metformin has

induced cytoprotective autophagy alleviating drug-activated apoptosis in these cells. This study implicated LKB1 (AMPK activator) independent autophagy induction with negligible effects on tumors of diabetic patients [28]. Analysis on human osteosarcoma cell lines delineated that metformin has induced cell cycle arrest, apoptosis and autophagy via reactive oxygen species-c Jun-N terminal kinase/ROS-JNK signaling pathway not clearly investigating AMPK/mTOR signaling pathway and not resolving the role of induced autophagy [29].

Regarding adjuvant therapeutic approach of AMPK/mTOR pathway activation via metformin, enhanced cisplatin antiproliferative effects in cholangiocarcinoma cells was presented [30].

Effect of metformin on glucose, lipid and nucleotide metabolism as a part of AMPK signaling network

Beside from protein metabolism inhibition, activated AMPK suppresses the Warburg effect including as a consequence aerobic glycolysis inhibition and glucose source deprivation for cancer cells. Since metformin is AMPK agonist, Faubert [31] suggested in the study that metformin application abolishes Warburg effect and therefore inhibits tumor growth (**Figure 1**). Presumed mechanism is that metformin activates AMPK followed by mTOR inihibiton and suppression of HIF-1 alpha transcription factor (hypoxic inducible factor), which is partially activated by mTOR. HIF-1 alpha stimulates the expression of genes involved in glycolysis [32].

Amount of phospholipids and higher fatty acids is elevated in different types of cancer [33], since cancer cells use them for proliferation and growth. Their synthesis is regulated via fatty acid synthase (FAS) enzymes. It has been described that in colorectal cancer metformin-activated AMPK consequently dysregulates lipid metabolism suppressing *in vitro* cancer stem cells and *in vivo* on mouse model preventing tumor growth [34]. AMPK activation by metformin downregulated FAS expression in aggresive metastatic cervical cancer and reduced viability in prostate cancer cells [35, 36]. Importantly, ex vivo study [37] shown that combination of metformin and salicylate derivatives activates AMPK and synergizes in reducing the survival of prostate and lung cancer through inhibition of de novo lipogenesis (**Figure 1**).

Elevated DNA synthesis is also specificity of cancer cell metabolism. However there is a few data dealing with metformin control of DNA synthesis. Current studies have shown that metformin depending on AMPK activity hinders de novo synthesis of nucleotides crucial for repair and synthesis of DNA molecules in several breast cancer cell lines [17] and pancreatic ductal cells *in vitro* [38]. Also, metformin induced nucleotide triphosphate depletion specifically in cancer stem cells [39]. In 5-Fu (5-fluorouracyl) resistant colon cancer cell line metformin preferably altered the DNA damage response and DNA replication but it persist a question of AMPK requirement in this model [40]. We don't ignore likelihood that noticed metformin alternations of DNA metabolism are mitochondria dependently since Kreb's cycle disturbation might interfere with DNA synthesis.

Different outcomes of AMPK-dependent metabolic regulation activated by metformin

Quoted studies signify anticancer role of metformin via AMPK activation, promoting changes in protein, glucose, lipid and nucleic acid metabolism hence depriving cancer cells of the essential substrates necessary for their growth and proliferation. A recent research however, reported that pharmacologic inhibition of AMPK curbed proliferation of prostate cancer cell lines [41]. This study hinted that outcome of metformin effect on AMPK activation depends on the cell environment. Namely, activation of AMPK with metformin refers to the glucose concentration in the extracellular environment [42]. For example, AMPK activation by metformin in the presence of a regular glucose concentration in the cellular environment will lead to antiproliferative effects [43] and related result was obtained by metformin and glucose reduction, decreasing the migratory ability of hepatocellular carcinoma cells [44]. Conflictingly, a study has shown that in low glucose environment conditions AMPK activation improves renal cancer cell proliferation [45]. Moreover, an in vivo study showed that in mice on a high-energy starvation metformin reduced tumor growth and away from impact in mice on control diet [46]. A related study posed that AMPK activation, as a result of lower ATP/AMP ratios in tumor microenvironment, promotes cancer cell residue under stressful metabolic conditions [47]. In light of cited study it is not surprisingly that in similar research AMPK acti-

vation by metformin promotes survival of dormant ER(+) breast cancer cell population [48].

Important fact to note is a particular outcome of AMPK activation relies not only on the extracellular environment but also on the condition of signaling pathways in the cell, e.g. it is reported that reactivation of AMPK in lung adenocarcinoma cells that do not have its upstream regulator LKB1, protects lung cancer cells from death caused by glucose starvation [49]. Also, cellular condition is described albeit mitochondrial ATP production is reduced by metformin and additionally cancer cell has activity failure AMPK or p53. In afore mentioned situation cancer cells are not producing sufficient energy and lack balance energy consumption finally suggesting energy crisis and cell death, as well as tumor suppression [46, 50, 51].

Although it is discernible that metformin can restrict anabolic course in cancer cells by activating AMPK, causing numerous AMPK consecutive pathways activation and in the end leading to cell death, it is also evident that during the later stages of malignant tumor development, AMPK activation supports the survival of tumor cells. Conceivable interpretation is that AMPK induction allows the cancer cell to adapt to the metabolic stress that exists intracellular and likewise in the tumor micro-environment.

Since immense importance of AMPK role in autophagy modulation we would suggest that metformin in some conditions activates cytoprotective autophagy as mechanism of tumor cells survival and thus scrutiny in establishing therapeutic strategy of metformin as a single agent in cancer therapy.

Effect of metformin on AMPK-dependent processes in oncogene and tumor suppressor signaling pathways

In addition to metabolic modifications, there are diverse studies proposing that AMPK activation induced by metformin contributes to proliferation and survival regulation by interacting with oncogenes and tumor suppressors.

MAPK pathway

For instance AMPK signaling controls cellular metabolism, whereas the mitogen activated protein kinases-MAPK signaling regulates cellular proliferation, differentiation and survival. Nonetheless, many studies have determined that these specific signaling pathways have sophisticated interplays in physiological and pathological processes [52].

It was shown that metformin inhibited proliferation, promoted apoptosis, reversed and delayed acquired resistance to Epidermal growth factor receptor tyrosin kinase inhibitors/EGFR TKIs in EGFR-mutant lung cancer [53]. These effects of metformin are associated with AMPK activation resulting in suppression of downstream extracellular signal-regulated kinases/Nuclear factor-kappa B-ERK/NF-κB signaling. Customary, mutated kirsten rat sarcoma virus oncogene/KRAS in colorectal cancer determinates excessive glycolysis having as a consequence high ATP production and AMPK inhibition [54]. Therefore AMPK inhibition is associated with decreased response of colorectal cancer cells to anti-EGFR antibody therapy [54]. On the contrary AMPK activation by metformin could suppress the KRAS mutation effect on anti-EGFR antibody resistance in colorectal cancer cells (**Figure 2**).

Metformin may be particularly effective when used in combination with small molecule kinase inhibitors that suppress glycolysis. In melanoma cells, the efficacy of the protein kinase inhibitor v raf murine sarcoma viral oncogenic homologue B1/BRAF has been shown to be limited by the induction of compensatory oxidative phosphorylation [55]. Although there are many oxidative phosphorylation inhibitors known to be toxic in cancer cells, the desirable safety profile of metformin implies this drug may have therapeutic potential [56].

Data from these studies implicated novel and further molecular rationale and preclinical data supporting combination of metformin with EGFR TKIs and small kinase inhibitors to treat cancer patients with mutations in MAPK kinase pathways.

Wnt/β-catenin pathway

One of the oncogenic pathways modulated by metformin is Wingless and Int-1 portmanteau-Wnt/β-catenin network pathway. This is an evolutionarily preserved and versatile signaling that is known to participate in physiologic conditions and besides in a wide variety of human diseases. Irregular activation of this pathway

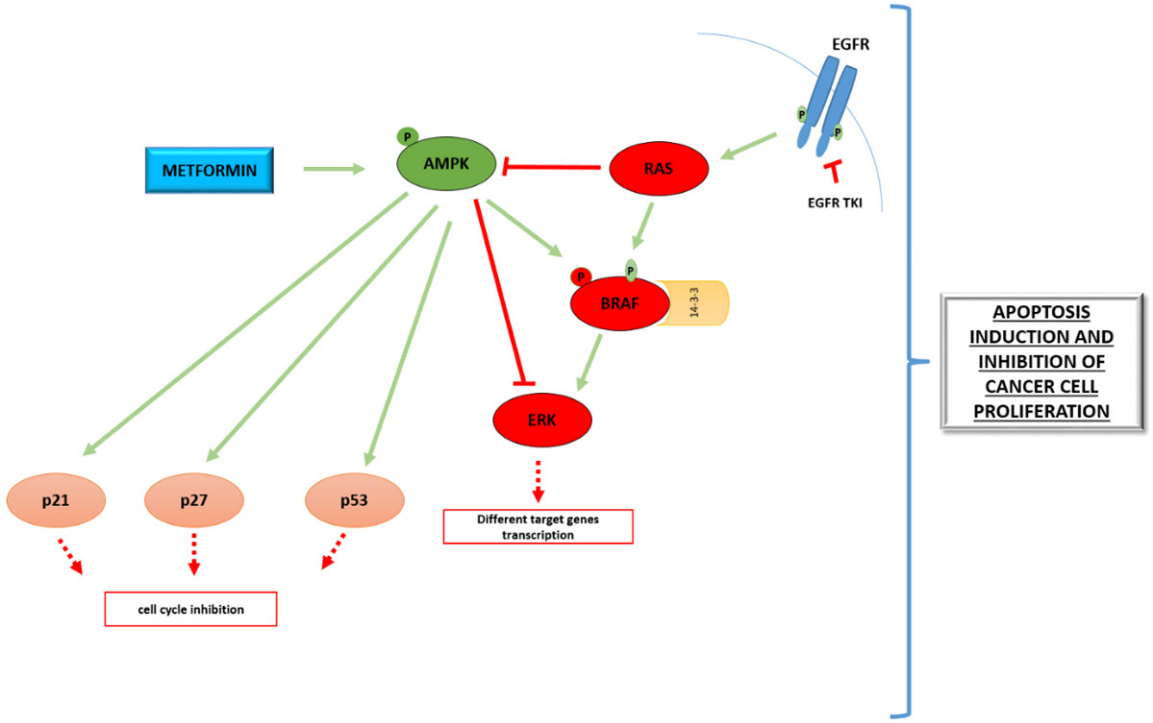

Figure 2. AMPK-dependent effect of metformin on evident oncogenes and tumor suppressor genes. Oncogenic MAPK pathway (red molecules) and tumor suppressors (light pink molecules) described in review are marked. Green arrows represent activation, while red lines show inhibitory interaction. Red squared boxes represent inhibition of pointed signaling regulation.

generates the accumulation of β-catenin in the nucleus and stimulates the transcription of different oncogenes such as *c-Myc* and *Cyclin D-1* [57].

It was recently shown that metformin can inhibit proto-oncogen activity of β-catenin through non-canonical AMPK/phosphatidylinositol 3-kinases/Protein kinase B-AMPK PI3K/Akt pathway phosphorylation in colorectal cancer cells [58] and to overcome breast cancer cell growth by downregulation of upstream Wnt/β-catenin signaling pathway regulators [59] (**Figure 3**).

Compelling study has demonstrated that AMPK activation by metformin can suppress β-catenin-dependent Wnt signaling by cytoplasmic sequestering of β-catenin via AMPK, further reducing cell proliferation in colon carcinoma cells [60].

These studies reported a novel mechanism of antineoplastic effect for metformin, and indicate for the upgrading of new antineoplastic drugs having the AMPK-Wnt/β-catenin pathway as therapeutic goal (**Figure 3**).

Foxo transcription pathway

Metformin stimulates another crucial pathway in cancer development and progression-tumor suppressor Foxo signaling Forkhead box. FoxO transcription factors are tumor suppressors in human cancers and recent studies have noted that besides their prevailing functions in cell cycle arrest initiation and cell death stimulation FoxOs can also coordinate cancer metabolism [61].

AMPK specifically phosphorylates FoxO3 on six different residues but these phosphorylations do not alter FoxO3 cellular localization, but may control FoxO3 binding on the target genes regulating energy stress [61].

It was recently suggested that metformin inhibits cancer cell growth in different types of cancer: endometrial, hepatocellular cancer cell lines, and *in vivo* on rat breast carcinoma

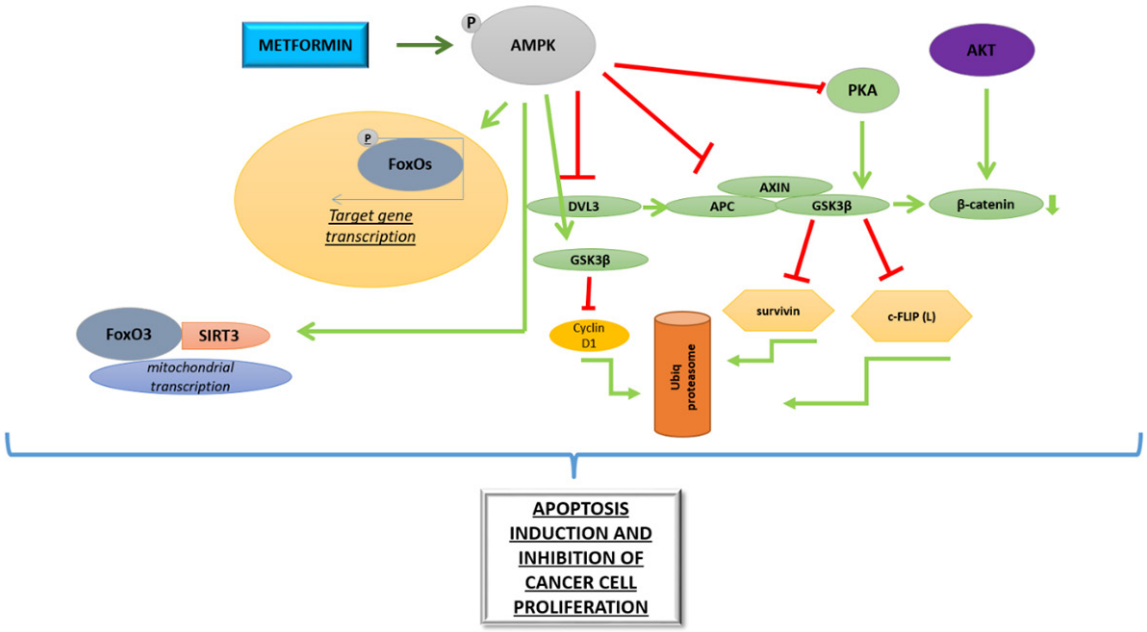

Figure 3. AMPK-dependent effect of metformin on atypical oncogenes and tumor suppressor genes. Green arrows represent induction, while red lines show suppression of phosphorylation or mutual molecules interaction and proper mechanisms.

(Walker-256) by AMPK-FOXO1/3 signaling pathway activation [62-64] (**Figure 3**).

GSK-3 pathway

Intriguing signaling that cannot been exclusively attributed to oncogenic or tumor suppressor is glycogen synthase kinase 3/GSK-3β pathway.

GSK-3β aside from modulating glycogen metabolism serves as tumor suppressor or oncogene depending on cellular conditions [65].

Group of authors demonstrated in non-small cell lung carcinoma that metformin-induced apoptotic cytotoxicity has promoted survivin and c-FLIP(L) destabilization depending on AMPK/protein kinase A-PKA/GSK-3β-axis [66, 67]. Metformin has caused in ovarian cancer cells cyclin D1 degradation via AMPK/GSK3β signaling axis and ubiquitin/proteasome pathway and attained anticancer effect (**Figure 3**).

Cell cycle regulators

Several studies reported AMPK-dependent effect of metformin on pivotal oncogenes and cell cycle regulators. It has been defined that metformin diminished the level of c-MYC oncogenes in an AMPK-dependent manner in the breast cancer cell line [68]. It is also delineated that AMPK sustained conformation and enhance production of tumor suppressor p53 [69, 70]. Metformin potentially through an AMPK activation, endorsed generation of p53, p21CIP1 and p27KIP1 in leukemic cell line and esophageal squamous cell carcinomas [71, 72]. Moreover metformin augmented expression of AMPK-dependent cell cycle regulators p21 and p27 in cervical cancer cell line [73] (**Figure 2**).

The complexity of oncogenic/tumor suppressor regulation proposes multiple molecular levels which can be modified by metformin influence on AMPK thus recommending jointed targeting of important molecular nodes in cancer cells.

AMPK independent effect of metformin on diverse signaling pathways in cancer cell

AMPK-independent processes that may explain the anti-cancer effects of metformin have also been described. These mechanisms include AMPK independent variation of diverse molecular networks in the cancer cell.

Exceptionally, metformin can inhibit the activity of mTOR in an AMPK-independent manner in prostate cancer cell line [74] causing cell cycle arrest persuading expression of a metabolic regulator tumor suppressor Redd1, previously described as a negative regulator of mTOR [74]. Though in glioblastoma metformin-engender AMPK-reliant Redd1 production [1]. Intriguing metformin can constrain mTOR omitting AMPK in a process related to amino acid removal induced starvation [75]. There is evidence provided by experiments on the prostate cancer cell line showing that metformin can inhibit critical cell cycle regulator cyclin D1 [76] and comparably in breast cancer, modulating cell cycle by cell division cycle 42-CDC42 suppression [77].

Numerous studies have demonstrated that metformin increases ROS independently of AMPK and thus far induces apoptosis in osteosarcoma, colon cancer, ovarian cancer, renal cancer, hepatocellular carcinoma and melanoma cell lines [29, 70, 78-81]. ROS elevation by metformin can also augment transitory decline in colon cancer cell lines proliferation [82], or even ROS dependent cytoprotective autophagy in breast cancer in vitro [83]. Contrary, *in vitro* study on melanoma [70] concluded that ROS increase induced by metformin stimulated cytotoxic autophagy. Likely, it has been recommended that interference with anti-oxidant hemeoxygenase-1 (HO-1) expression is a potential therapeutic approach to sensitize tumors to chemotherapy and radiotherapy. Results from Do [84] indicate that metformin suppresses HO-1 mRNA and protein expression in human hepatic carcinoma HepG2, cervical cancer HeLa, and non-small-cell lung cancer A549 cells, attenuating rapidly accelerated fibrosarcoma-ERK-nuclear factor erythroid 2-related factor 2/Raf-ERK-Nrf2 signaling-separate of AMPK. Further this study illustrates how metformin curtails MAPK pathway excluding AMPK.

Metformin in cancer cell regulates numerous signaling pathways and there are also studies describing apoptosis activation on AMPK independent manner. A study performed on squamous cell esophageal carcinoma showed that metformin induced apoptosis and autophagy excluding AMPK by interrupting antiapoptotic Stat3 (signal transducer and activator of transcription)/Bcl2 (B-cell lymphoma 2) signaling pathway resulting in reduced tumor growth [85]. Yang has implicated novel role of metformin in induction of endoplasmatic reticulum (ER) dependent apoptosis [86]. The latest study presented evidences that intracellular acidification by metformin in solid tumors triggered the unfolded protein response to induce the global transcriptional repressor (DNA Damage Inducible Transcript 3)/*DDIT3*, known to block oncogenic Wnt signaling pathway [87]. Further, metformin coordinates glucose metabolism omitting AMPK as blocking glucose uptake in a lung cancer cell line model through direct allosteric inhibition of hexokinase-II [88]. Removal of this energy source causes depolarization of mitochondria and results in activation of apoptosis. A similar finding was observed in the breast cancer cell line [89].

Part of metformin controled AMPK-dependent pathways can also be modified on AMPK independent manner as it is described upper: mTOR inhibition, regulation of cell cycle, MAPK pathway modulation and inhibition of Wnt signaling pathway.

Metformin has also been shown to increase the sensitivity of various types of cancer cells to common chemotherapeutics including cisplatin, paclitaxel, carboplatin, and doxorubicin [90-92]. However, it should not be overlooked that metformin may antagonize the cytotoxic effect of cisplatin in glioma, neuroblastoma, fibrosarcoma and leukemia cell lines [93] via AMPK-independent activation of the Akt signaling pathway in glioma cell line. This question is rather intriguingly since there is the recent study implying that metformin augments the response of CRC (colorectal cancer cell line) cells to cisplatin through ROS-mediated PI3K/Akt signaling pathway [94].

Metformin in cancer clinical studies

Since 2005, when the Evans study appeared, there has been a sharp increase in interest in metformin in the prevention and treatment of cancer. For clearness, the clinical studies finished after 2015 year in this review are presented in tables (**Tables 1** and **2**). Interest in the use of meformin in the treatment of cancer is not decreasing, thus in 2016, 15 studies that were not completed were started, in 2017-14 studies were started, which are ongoing, and in 2018, 14 studies that were started and still not ended.

Table 1. Masked clinical studies completed after 2015

STUDY	A Randomized Phase II, Double Blind Trial of Standard Chemotherapy With Metformin (vs. Placebo) in Women With Metastatic Breast Cancer Receiving First to Fourth Line Chemotherapy
NCT NUMBER	NCT01310231
STATUS	Completed
PARTICIPANTS	40 participants, 18-75 years, sex: female
PERIOD	February 2011-March 2021
INTERVENTION	Drug: Metformin Metformin vs. placebo 850 mg bid in addition to standard chemotherapy (containing anthracyclines, platinum, taxanes or capecitabine; first or second line).
CANCER TYPE	Metastatic breast cancer
OUTCOME	In this population metformin showed no significant effect on RR, PFS or OS. These results do not support the use of metformin with chemotherapy in non-diabetic MBC patients.
STUDY	Phase II Study of Metformin in a Pre-prostatectomy Prostate Cancer Cohort
NCT NUMBER	NCT01433913
STATUS	Completed
PARTICIPANTS	20 participants, sex: male
PERIOD	Sep 2011-May 2018
INTERVENTION	Metformin hydrochloride vs. placebo both PO 4-12 weeks
CANCER TYPE	Adenocarcinoma of the Prostate: Recurrent Prostate Cancer, Stage I Prostate Cancer, Stage IIA Prostate Cancer, Stage IIB Prostate Cancer
OUTCOME	No differences between the biomarker expression in the prostatectomy tissue or pre to postintervention changes in serum biomarkers (prostate-specific antigen, insulin, insulin-like growth factor-1, insulin-like growth factor binding protein 3, sex hormone-binding globulin, and testosterone) or tissue biomarkers of proliferation apoptosis, cell cycle regulation, and mTOR inhibition
STUDY	A Phase II, Randomized, Placebo Controlled Study to Evaluate the Efficacy of the Combination of Gefitinib and Metformin in Patients With Locally Advanced and Metastatic Non-Small-Cell-Lung-Cancer
NCT NUMBER	NCT01864681
STATUS	Completed
PARTICIPANTS	97 participants, 18-75 years, sex: all
PERIOD	May 2013-June 2018
INTERVENTION	Gefitinib + metformin vs. gefitinb + placebo
CANCER TYPE	Treatment-naïve stage IIIB-IV with EGFR mutation in NSCLC
OUTCOME	incorporation of metformin into standard gefitinib therapy did not prolong PFS or OS in treatment naïve nondiabetic patients with EGFRm NSCLC, neither did it elicit an increase in response to gefitinib
STUDY	Placebo Controlled Double Blind Crossover Trial of Metformin for Brain Repair in Children With Cranial-Spinal Radiation for MedulloblaStoma
NCT NUMBER	NCT02040376
STATUS	Completed
PARTICIPANTS	30 participants, 5-21 years, sex: all
PERIOD	January 2014-May 2019
INTERVENTION	Metformin and placebo doses will be 500 mg/m^2 po daily given in 2 doses for one week and if there are no concerns increased to 1000 mg/m^2 po daily given in 2 doses for the rest of the 12 week trial. The investigators will use the closest dose according to body surface area (250-500-750-1000) BID.
CANCER TYPE	Brain tumor
OUTCOME	Evidence that a clinical trial examining the effects of metformin on cognition and brain structure is feasible in long-term survivors of pediatric brain tumors and that metformin is safe to use and tolerable in this population
STUDY	A Randomized, Phase II, Double-blind, Placebo-controlled, Multicenter, 2x2 Factorial Design Biomarker Tertiary Prevention Trial of Low-dose Aspirin and Metformin in Stage I-III Colorectal Cancer Patients. The ASAMET Trial
NCT NUMBER	NCT03047837
STATUS	Unknown
PARTICIPANTS	180 participants, 18-80 years, sex: all
PERIOD	March 2017-February 2019
INTERVENTION	Drug: Aspirin (ASA) + Metformin (MET) Arm D (experimental arm) Treatment: active ASA + active MET Dose: 100 mg, 1 tablet daily + 850 mg, 1 tablet twice a day (BID) Duration: 12 months Drug: ASA Arm C (experimental arm) Treatment: active ASA + placebo MET Dose: 100 mg, 1 tablet daily + 1 tablet twice a day (BID) Duration: 12 months Drug: MET Arm B (experimental arm) Treatment: placebo ASA + active MET Dose: 1 tablet daily + 850 mg, 1 tablet twice a day (BID) Duration: 12 Arm A (control arm) Treatment: placebo ASA + placebo MET Doses: 1 tablet daily + 1 tablet twice a day (BID) Duration: 12 months

CANCER TYPE	Stage I, II, or III primary colorectal cancer
OUTCOME	A favorable biomarker modulation by aspirin and metformin may provide important clues for a subsequent phase III adjuvant trial aimed at preventing second primary cancer, delaying recurrence and improving prognosis in patients with CRC.
STUDY	Effect of Metformin for Deceasing Proliferative Marker in Endometrial Cancer Cells: A Randomized Double Blind Placebo-controlled Trial
NCT NUMBER	NCT03618472
STATUS	Completed
PARTICIPANTS	50 participants, child, adult,older adult, sex: female
PERIOD	August 2018-January 2020
INTERVENTION	Drug: Metformin Hydrochloride 850 MG Regular strength metformin (850 mg/tab) vs. placebo
CANCER TYPE	Endometrial cancer who undergoing complete surgical staging
OUTCOME	Metformin administration reduced Ki-67 expression and reduced grade in endometrial tumor when given for 4 weeks before hysterectomy

Table 2. Unmasked metformin clinical studies ended after 2015

STUDY	A Phase II Evaluation of Metformin, Targeting Cancer Stem Cells for the Prevention of Relapse in Patients With Stage IIC/III/IV Ovarian, Fallopian Tube, and Primary Peritoneal Cancer
NCT NUMBER	NCT01579812
STATUS	Completed
PARTICIPANTS	38 participants, 18-80 years, sex: female
PERIOD	April 2012-May 2018
INTERVENTION	Metformin
CANCER TYPE	Ovarian, Fallopian Tube, and Primary Peritoneal Cancer
OUTCOME	Metformin therapy was associated with better-than-expected overall survival, supporting the use of metformin in phase III studies
STUDY	Pilot Study of Metformin in Head and Neck Squamous Cell Cancer and Its Effects on Stromal-epithelial Metabolic Uncoupling
NCT NUMBER	NCT02083692
STATUS	Completed
PARTICIPANTS	50 participants, 18-80 years, sex: all
PERIOD	March 2014-October 2016
INTERVENTION	Metformin
CANCER TYPE	Head and Neck Squamous Cell Cancer
OUTCOME	Metformin treatment may favorably alter the immune TME (tumor microenvironment) in HNSCC independent of HPV status.
STUDY	Prospective Evaluation of Clinical Safety of Combining Metformin With Anticancer Chemotherapy
NCT NUMBER	NCT01442870
STATUS	Completed
PARTICIPANTS	105 participants, 18-79 years, sex: all
PERIOD	September 2011-May 2017
INTERVENTION	Metformin
CANCER TYPE	Solid tumor
OUTCOME	This is the largest phase I study of metformin combined with chemotherapy, which suggests that metformin can be given safely with chemotherapy, and offers a platform for future studies. Post-metformin increase in AMPK phosphorylation may potentially explain lack of disease progression in nearly half of our patients.

Metformin, cancer and immunity

It is of quite relevance to have in mind that human immune system defend us not only from foreign pathogens but also from cancer considering cancer prevention, development, and metastasis [95]. In a cancer growth inflammation is comparable to double-edged sword.

At the onset of tumorigenesis, inflammation carries crucial function in abolishing transformed cells as an essential part of innate immunity, as well presenting cancer antigens for adaptive immunity [95]. Contrarily, chronic inflammation is recognized to raise the prevalence and malignancy in various cancer models [96]. Not long ago it has been described anti-inflammatory function of metformin in certain disease models. It was determined that metformin diminished inception of multiple sclerosis, control-case studies on T2D patients proposed that metformin hampers cardiovascular diffi-

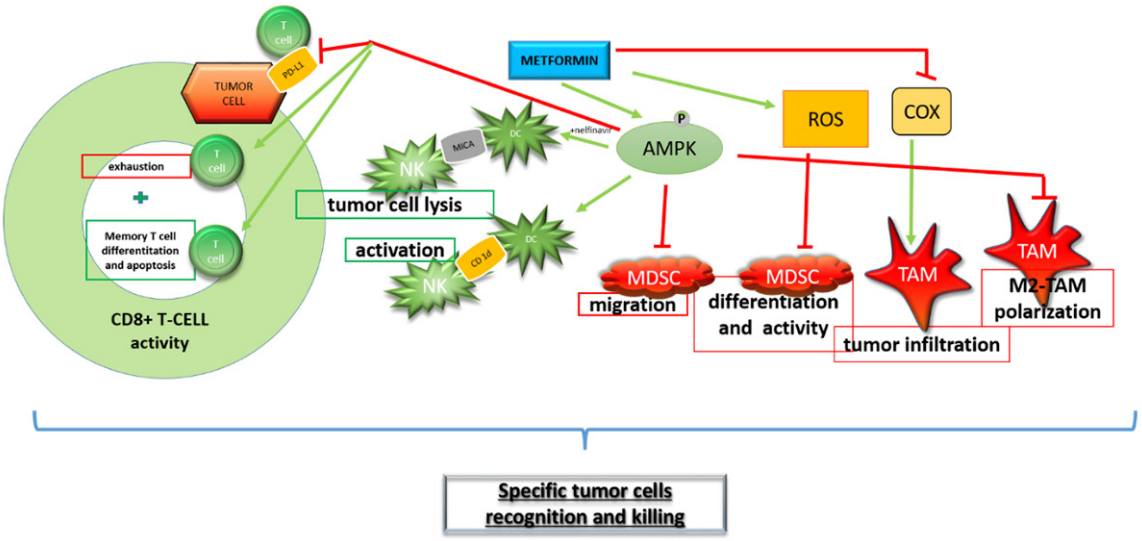

Figure 4. Potential role of metformin in cancer cell killing by immune system regulation. Cells filled red promote tumor survival, while green filled cells are involved in tumor cells killing. Green arrows represent activation of molecules or cells depicted on **Figure 4**, whereas red lines describes inhibitory interaction between metformin and presented molecules or cells. Red squared boxes show down regulation of specific process by metformin and green squared boxes describe process possibly up-regulated by metformin.

culties [97] and there is also perspective for metformin as treatment in inflammatory skin disorders [98]. So far it was implied that metformin regulates encouraging disease outcome, diminishing pro-inflammatory cytokines amount [99]. These findings have opened a new query focusing metformin as a possible therapeutic in immune-oncology.

The permanent tumor rescue phenomena involves natural killer cells (NK), tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs) and CD8+ cytotoxic T lymphocytes (CTL).

Metformin promotes NK-cells activation

NK cells have crucial function in the innate immune response of the host and are particularly responsive to metastatic cells or certain hematological cancers [100]. The processes by which NK cells detect cancerous cells remain obscure, although CD1d-mediated presentation by APC of glycolipid antigens to NK cells [101] and by major histocompatibility complex class I related chain A (MICA) (a non-classical HLA molecules) [102] has been evidenced. Since AMPK has important role in lipogenic pathways regulation, pretreatment with different pharmacological compounds among them and metformin, appropriately augmented CD1d and MICA mediated NK-cell activation [99]. These data propose that addressing lipogenic cellular metabolism in NK cells may provide an original target of innate immune responses stimulation (**Figure 4**).

Metformin shifts macrophage polarization

Macrophages can be classified into two main subsets according to their activation state, classically-activated M1 and alternatively-activated M2 [103]. TAMs, for nearly all types of cancer appear the M2-like subset. TAMs make specific cytokines thereupon restricting the antitumor immune response and augmenting tumor growth [103]. Several studies reported that metformin in lung, breast, prostate, osteosarcoma and diverse cancer models in murine alters TAM polarization form M2- to M1-like phenotype generally by switching cytokine production distinct for each subset, preventing the infiltration of TAMs and inducing the metabolic shift from M2 to M1-like TAM phenotype [103] (**Figure 4**).

TAM polarization has relevance in cancer immunity, thus it could extend a novel benefit for the supplementation of metformin as component of an immunotherapy procedure.

Metformin inhibits MDSCs activity

A significant immunosuppressive cell group that assembly in tumor-bearing patients are MDSCs that can block T cells function and reinforce tumor immune escape. *In vitro* and *in vivo* analysis described that metformin curtailed MDSC migration, abolished MDSC inhibitory activity and evoked Th1 and CTL feedback in murine colon cancer CT-26 cell-transplanted mice [99]. Delineated metformin effects are AMPK-dependent, imply inhibition of different AMPK substrates and subsequent restraining of human MDSC mediated immunosuppression (**Figure 4**).

Effect of metformin on CD8+ CTLs

Cancer cells provoke recurrent T cell receptor-TCR stimulation with tumor antigens and further continue in constantly deterioration of CD8+ CTL capacity to secrete interleukine-2/IL-2, tumor necrosis factor alpha/TNFα, and interferon gamma/IFNγ, subsequently undergoing apoptotic removal in a mechanism accepted as immune exhaustion [104]. Immune exhaustion is characterized by phenotypic modifications in CD8+ CTL cells, simultaneously with the expression of exhaustion markers like programmed cell death protein/PD-, and its high expression is noticed in several types of cancer. This observation is linked with poor prognosis [103]. Accordingly, improved CD8+ CTL activity versus cancer cells could be reached by retraction of PD-L1 impeding signal (**Figure 4**). Actually, there are several investigations validating significance of metformin in AMPK-dependent manner on PD-L1 levels starting from blocking of PDCD1 gene transcription, PD-L1 phosphorylation and subsequent disintegration in endoplasmic reticulum (ER), decline of membrane-bound PD-L1 in different cancer models [103]. Further, data obtained on various syngeneic animal models propose that fusion of metformin and Anti-CTLA-4 therapy, has the efficiency to improve immunotherapy [103]. Thus, these studies indicate that metformin can reinforce CD8+ CTL feedback by diminishing PD-L1 level (**Figure 4**).

The effect of metformin in metabolic adjustments of T-cells

It has already been presented metformin's role in cancer cell metabolism thus recent research explore effect of metformin on metabolic adaptation of T-cells in TME.

Considering that main regulators in energy sensing and mitochondrial function are AMPK and mTOR, well known downstream targets of metformin, there are studies proposing the effect of metformin on AMPK/mTOR axis in metabolic reprogramming of depleted CD8+ CTL in TME. These studies presumed that metformin by metabolic reprogramming favored CD8+ CTL activity, CD8+ memory T-cells generation and shift of T-cell central memory cells (TCM) to CD8+ T cell effector memory cells (TEM) [105]. Also, generation of the continuous antitumor immunity might be supported by negative influence of metformin on Ti-Treg [106].

These functions of metformin could have potential to be addressed for enhancing vaccine efficacy and antitumor immunity.

Possible function of metformin in cancer immunotherapy

Perhaps an original therapeutic concept is immunotherapy that could implement incomparable advantage to certainly amend the treatment of various diseases, including cancer. Current antitumor drugs are generally ambiguous and also harmful to normal cells. Contrary, immunotherapeutics facilitate the immune system to specifically recognize and kill tumor cells. However exhaustion of immune system in TME, imposes request for immune therapy breakthrough. Immunotherapy has the capability with a many recent technological upgrades in physicochemical and molecular biology field to become accepted as cancer treatment.

Form of phototherapy including light and photosensitizing chemical substance, applied in combination with molecular oxygen is referred as photodynamic therapy (PDT). Chemical damage of tumor cells can be obtained if enough oxygen is present to produce ROS by PDT. Likewise, PDT augments the interferon γ (IFN-γ) generation and stimulates the antitumor immunity of T cells. Overlooked consequence of boosted IFN-γ production is important increase of the programmed death-ligand 1 (PD-L1) expression on tumor cell membrane committing inhibition of CD8+ CTL cell function. New research reported structure as a two-in-one nanoplatform (IR775@Met@Lip) designed

by packing metformin (Met) and IR775 into a clinically practical liposome. Notably, nanoplatform displayed reduced PD-L1 expression, alleviated T cell exhaustion, conversed tumor hypoxia and successfully abolished both the primary and abscopal tumor growth in bladder and colon cancers, respectively. Aforementioned complex is considerably promising to become a feasible cancer therapy approach [107].

Developing branch of photodynamic therapy (PDT) is photothermal therapy (PTT). This concept proposes utilizing electromagnetic radiation (most often in infrared wavelengths) to move the sensitizer to an excited state where it then releases vibrational energy-heat that kills the targeted cells. Current studies also provided longer wavelength light application, which has less energy and therefore is less detrimental to other cells and tissues. Photothermal therapy (PTT) does not demand oxygen to affect the target cells or tissues contrary to photodynamic therapy.

Group of researchers proposed tumor vaccine vector (TA-Met@MS) comprised of tumor antigen (TA), metformin (Met) and Hollow gold nanospheres (HAuNS) in poly (lactic-co-glycolic acid) (PLGA) microspheres. NIR light-mediated photothermal effect can contribute to a pulsed-release behavior of TA and Met from the microspheres. Primary T cell expansion, contraction and stimulated production of effector T cells at the early immunization stage can be managed by released TA. As a result of AMPK activation controlled by metformin the metabolic scheme in the cells is then shifted from glycolysis into fatty acids oxidation (FAO). FAO can increase T cell survival and assist the differentiation of memory CD8+T cells. For upgrading cancer prevention and therapy this study may give a useful consideration to develop tumor vaccine [108].

Ongoing research in immunotherapy focused on creation the cell membrane-based biomimetic nanoparticles (NPs) constructs, to improve therapeutic and imaging applications. In these constructs the physicochemical properties of the NP are retained, whereas camouflaged complex components of a natural cell membrane should endorse the NPs with many fascinating biological functions. Lately, cancer cells and a variety of cells omitting red blood cells (RBCs), including platelets, immune cells (e.g., macrophages) have been manipulated to restore membrane materials. To support synthetic NPs with multifunctional and sophisticated cell-like functions single cell membrane appliance could be remodeled, by hybrid cell membrane-coating construction. Further, as an encouraging tool to attain multi-modal cancer therapy nano drug co-delivery system has appeared.

To analyse this principle metformin and siRNA Fibrinogen-like protein 1/FGL1 were encapsulated in PLGA to form a basis, following coating with a hybrid biomimetic membrane from macrophages and cancer cells to form a multiple-targeting biomimetic nanoplatform. To promote the endosomal/lysosomal evasion of the encapsulated siRNA for adequate cytosolic siRNA delivery this nanoplatform was created as pH-activated CO2 gas-producing nanoplatform (MC-PLGA@Met-CO2/siFGL1 NPs).

Important synergistic therapeutic efficacy against breast cancer *in vitro* and *in vivo* was presented with a PD-L1/programmed death 1 signaling restriction and FGL1 gene silencing. Futhermore, metformin stimulated M1-type differentiation of tumor-related macrophages diminishing tumor hypoxia and certainly developing the microenvironment immunosuppressive for tumor [109].

This results implied plausible position of MC-PLGA@Met-CO2/siFGL1 NPs nanoplatform in breast cancer immunotherapy application.

We suggest that these recently published papers implemented the role of metformin in state-of-the-art technology improvement in cancer immunotherapy.

Conclusion

Metformin is a commonly prescribed antidiabetic drug showing diverse anticancer effects *in vitro* and *in vivo*. The advantage of metformin in cancer therapy has to be validated in human pharmacodynamic, pharmacokinetic and phase II clinical studies especially in non-diabetic patients. The complexity of activated/inhibited signaling pathways in cancer cells during metformin administration in different in vitro/in vivo context may be a limiting factor when using this drug as possibly plain anticancer therapy.

Besides considering metformin as a single agent in cancer therapy, metformin might be used in appropriate combinations with targeted therapies taking into account their limited success. Physicians should be aware of possible inhibitory effect of metformin along with conventional chemotherapeutics application.

Recently, novel role of metformin in regulating cancer immunity has emerged. Intriguingly about this function of metformin is modulation of precise AMPK-dependent mechanisms shared between cancer cells and immune cells, but exerting in most instances favorable opposite effect: cancer cell killing and improvement of immune system cells function. Therefore, we imply that original approach in investigation of combined metformin-immunotherapy in cancer treatment could bridge the gap between basic molecular biology research and future clinical studies.

Acknowledgements

The work was supported by The Ministry of education, science and technological development of Republic of Serbia (No. 451-03-9/2021-14/200007 and No. 451-03-9/2021-14/200110). We acknowledge the critical discussions and suggestions of Prof Dr. Vladimir Trajkovic during manuscript writing.

Disclosure of conflict of interest

None.

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