Published in final edited form as:

Biomed Pharmacother. 2014 May ; 68(4): 493–496. doi:10.1016/j.biopha.2014.03.012.

NMDA receptors on the surface of cancer cells: Target for chemotherapy?

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Abstract

The mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a therapeutic target for many types of cancers. NMDA receptors regulate mTOR signalling activity; their inappropriate expression on several human cancer cell lines represents a potential therapeutic avenue to control dysregulated growth, division and invasiveness. Targeting these receptors with selective ligands (e.g., glycine_B site ligands) may be a less toxic and more tolerable approach than administering compounds acting at the mTORC1 complex itself, such as rapamycin and its derivatives. Thus, testing glycine_B site ligands in relevant *in vitro* and *in vivo* paradigms with established human cancer cells that express NMDA receptors on their surface could provide proofs of concept/principle that would encourage exploration of these and other “non-toxic” strategies. Interestingly, in some cancer models that express NMDA receptors on their surface, NMDA receptor antagonists, such as MK-801 (dizocilpine), were shown to possess anti-proliferative and anti-invasive effects, which conflict with hypotheses about promoting NMDA receptor activation as a cancer chemotherapeutic strategy. Whether NMDA receptor activation or antagonism is associated with anti-proliferative and anti-invasive effects may reflect differences between cancer cell lines in terms of the proteins associated with the NMDA receptors on their cell surfaces, which, in turn, could lead to different “downstream” effects on cascades of intracellular phosphorylations. Irrespective of whether activation or antagonism is associated with anti-proliferative and anti-invasive effects for specific types of cancer, data are emerging that support exploration of targeting NMDA receptors expressed on the surface of cancer cells as a therapeutic strategy.

Keywords

NMDA receptor; Cancer; mTOR; Chemotherapy

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Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

1. Introduction

1.1. NMDA receptor: pharmacology and regulation of mTOR signalling activity

The *N*-methyl-D-aspartic acid (NMDA) receptor, a glutamate-gated ion channel receptor, has been characterized on the surface of several cancer cell lines [2–5]. NMDA receptors are heterotetrameric integral membrane proteins constructed from constituent polypeptides that encircle a potential channel, whose opening is gated by the binding and cooperative interaction of L-glutamate and the obligatory glycine co-agonist at the cell's surface [6]. In addition to the binding of glutamate and glycine, channel opening is a highly regulated process that is influenced by the membrane potential, the extent to which intracellular domains are phosphorylated and a variety of other post-translational modifications, including nitrosylation, and concentrations of allosteric modulators, such as neurally-active steroids and polyamines [7–9]. Ordinarily, at the resting membrane potential of the cell, a hydrated magnesium ion occupies a site within the channel domain interfering with calcium ion flux, this magnesium ion blockade is relieved as the cell begins to depolarize, which is usually dependent on glutamate's stimulation of another class of ion channel receptor located on the cell surface in close proximity to the NMDA receptor (i.e., the AMPA receptor). NMDA receptor activation results in calcium ion entry and membrane depolarization. In addition to electrophysiological consequences of glutamate-gated calcium ion entry, there are a host of metabolic consequences related to the transient increase in calcium ion concentrations. Very interestingly, NMDA receptor activation affects signalling activity by the mammalian target of rapamycin (mTOR), a serine/threonine kinase, contained within the mTORC1 complex, which influences transcription and translation [10–12]. For example, NMDA receptor activation results in the negative regulation of cationic amino acid transporters within the cell's plasma membrane, which are responsible for arginine transport into the cell, causing their rapid internalization and lowering of intracellular levels of arginine. Lowered intracellular arginine concentrations are detected by “nutrient sensors” that lead to dampening of mTOR signalling activity [10]. Furthermore, NMDA receptor activation leads to the Ca^{2+} -dependent activation of calcineurin, a protein phosphatase, whose substrates include STriatal Enriched protein tyrosine Phosphatase (STEP). STEP, in turn, acts on phosphorylated ERK1/2, an extracellular signal-regulated kinase that is a driver of mTOR signalling (Fig. 1) [11,12]. Thus, NMDA receptor activation limits the duration of signalling by ERK1/2, which dampens mTOR signalling activity. Of interest to this hypothesis, dysregulated mTOR signalling activity is implicated in the pathogenesis of malignancy and metastasis, and is a chemotherapeutic target [1,13]. Unique combinations of NMDA receptor subunits and their splice variants constituting functional receptors on the cell's surface determine preferential interactions with intracellular proteins; properties of functional NMDA receptors at the cell surface may also influence intracellular signalling cascades [14–16].

1.2. NMDA receptor: regulation of cancer cell growth, division and invasiveness

NMDA receptor activation inhibits the outgrowth of keratinocytes in epithelialization processes, which may be relevant to suppressive effects on tumor invasiveness [14–16]. Thus, functional NMDA receptors that gate Ca^{2+} are expressed in keratinocytes of normal human skin [15]. Data suggest that these NMDA receptors play a role in contact inhibition

of keratinocyte cell growth, a process that is disrupted in cancer. Interestingly, whereas NMDA receptor activation inhibits keratinocyte outgrowth, expression of the NR1 NMDA receptor subunit, which is an obligatory subunit for forming functional NMDA receptors, was absent in a cutaneous squamous cell carcinoma. However, the reactive non-neoplastic epithelium surrounding the squamous cell carcinoma expressed the NR1 subunit. These data are consistent with a role for functional NMDA receptors and their activation in regulating keratinocyte differentiation, migration and malignant transformation [15]. Consistent with their suppressive roles in some tumors, gene expression profiling has also shown that expression of NMDA receptors is downregulated in samples of human primary glioblastoma multiforme tissues [14]. In fact, expression of NR2B subunits is low in a variety of cancer cell lines [3].

Transcriptional silencing of tumor suppressor genes can occur as a result of aberrant hypermethylation of their promoter regions, which increases the likelihood that the *MeCP2* protein, a methylated DNA binding protein, will bind to this region and recruit histone deacetylase (HDAC), leading to chromatin condensation [17]. *NMDAR2B* is emerging as a tumor suppressor gene; [14,17,18] hypermethylation of its promoter region and silencing of its expression was found in 12 out of 12 esophageal squamous cell carcinoma (ESCC) cell lines and in 55 of 61 (~90%) specimens of primary ESCC [17]. Interestingly, tumor suppressor activity of NMDA receptor activation could be inhibited in human ESCC cell lines by ifenprodil, a specific NMDAR2B antagonist. Expression of *NMDAR2B* mRNA was also absent or reduced in six gastric cancer cell lines (MKN1, MKN7, NKN74, AZ521, NUGC4 and NUGC3), relative to robust expression in normal stomach [18]. Moreover, this absent or reduced expression in these six gastric cancer cell lines resulted from transcriptional silencing due to methylation of CpG islands in the promoter region of *NMDAR2B*. Similarly, reduced expression of *NMDAR2B* and hypermethylation of its promoter were correlated in primary gastric tumors. Further, the relevance of *NMDAR2B* to growth suppression of gastric tumors was shown by transfecting four of the gastric cell lines with *NMDAR2B* expression plasmids causing its overexpression, which resulted in inhibition of their colony forming ability, relative to when the cell lines were transfected with vector alone [18]. All of the transfected cell lines expressed *NMDAR1* (the obligatory NR1 subunit of the tetrameric NMDA receptor complex); thus, they were all capable of making functional NMDA receptors. Again, *NMDAR2B* is expressed in normal stomach epithelial cells, but its expression was found to be absent and reduced in 14% and 77% of 35 gastric cancers, respectively [18]. Conceivably, targeting the functional NMDA receptor-gated channels in the 77% of gastric cancers with reduced and 9% of gastric cancers with normal levels of expression would have tumor suppressive effects. The authors also suggested that measuring methylation of the *NMDAR2B* promoter region might serve as a biomarker to assess tumor risk in normal tissue adjacent to a tumor, as well as a marker for early detection and monitoring of gastric cancers [18]. Further, transcriptional silencing of the *NMDAR2B* gene due to methylation was demonstrated in five out of nine non-small cell lung cancer (NSCLC) cell lines [16]. Moreover, 61% of resected tumor samples from 216 unselected patients with NSCLCs showed methylation of *NMDAR2B*, which influenced prognosis: poor prognosis in adenocarcinoma and better prognosis in squamous cell carcinoma.

Glutamate receptors, including the ionotropic NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors, participate in the regulation of the proliferation, motility and invasive growth of several different types of tumor cells [19]. In a calcium ion dependent manner, NMDA and AMPA receptor antagonists altered morphology, and inhibited cell division and migration of tumor cells (e.g., breast and lung carcinoma) grown *in vitro*. Importantly, the proliferation of human skin fibroblasts and bone marrow stromal cells were unaffected by NMDA and AMPA receptor antagonists. Moreover, tumor cells, including colon adenocarcinoma, astrocytoma, lung and breast carcinoma, neuroblastoma, rhabdomyosarcoma/medulloblastoma and thyroid carcinoma, but not human skin fibroblasts, stained immunohistochemically positive for the NR1 subunit of the NMDA receptor and GluR2/3 subunits of the AMPA receptor. The NMDA receptor antagonists that were tested and effective were non-competitive, open-channel blockers, which not only obstruct calcium ion conductance by binding to a hydrophobic channel domain, but also have allosteric modulatory effects on glutamate and glycine binding.

More recent experiments show that expression of ionotropic glutamate receptor subunits and metabotropic glutamate receptors are detectable in normal human skin fibroblasts, in addition to various human cancer cell lines [3]. Although the levels of expression are usually lower than that of human brain, there are exceptions; for example, expression levels of the *NR2BNMDA* receptor subunit in LS180, a human colon adenocarcinoma cell line, was estimated to be about 50% of levels in human brain [3]. The unique combination of receptor subunits will determine the pharmacological properties of ionotropic glutamate receptors; the heterogeneity of glutamate receptors is further increased by post-transcriptional modifications, resulting from RNA editing and alternative splicing of RNA. There are eight distinct isoforms of the NR1NMDA receptor subunit, and several splice variants of the NR2 and NR3 subunits. The existence of this heterogeneity in specific human cancers will facilitate the selective targeting of specific receptors on the surfaces of particular cancer cells with NMDA receptor agonists and antagonists that have anti-proliferative properties for these cells. Selective ligands for ionotropic glutamate receptors with specific receptor subunit combinations, including specific splice variants, are available. Further, at least in the case of oral squamous cell carcinoma, expression of the *NR1NMDA*, receptor subunit was associated with measures of tumor size, lymph node metastases and patient survival; these data are consistent with a regulatory role for NMDA receptors in tumor cell proliferation and invasiveness [20].

MK-801 (dizocilpine) and memantine, two non-competitive NMDA receptor antagonists, were shown to have concentration-dependent cytotoxic effects on human lung adenocarcinoma cells (A549) grown in vitro [2]. These cells were shown to express NR1 and NR2B NMDA receptor subunits. Moreover, in the A549 cell line, MK-801 decreased levels of immunoreactive phosphorylated ERK1/2 in a concentration-dependent manner without affecting immunoreactive levels of total ERK1/2. Further, culturing A549 with MK-801 was associated with decreased activation (i.e., phosphorylation) of the cAMP-response element binding protein (CREB) that, in turn, regulates transcription of many genes involved in the cell cycle, such as *p21*. [2] *p21* codes for a tumor suppressor protein, whose expression is upregulated in A549 cells as a downstream consequence of culturing these cells in the presence of MK-801. MK-801 also antagonized stimulating effects of epidermal

growth factor, insulin growth factor, and basic fibroblast growth factor on A549 cells. Additionally, MK-801 prolonged survival of severe combined immune deficient (SCID/SCID) mice injected with A549 cells into their peritoneal cavity and impaired the growth of neuroblastoma and rhabdomyosarcoma cells injected into the right flank of congenitally athymic, immunologically incompetent nude (nu/nu) mice. These data encourage exploration of drugs that target the NMDA receptor for their anti-proliferative and anti-invasive effects on specific cancer cell lines that express these receptors on their surface.

Human small cell lung cancer (SCLC) tumor cell lines (i.e., NCI-H345, DMS-53, NCI-H146, and NCI-H82) were shown to express functional NMDA receptors; additionally, tissue sections of eight of 10 SCLC tumors stained positive with anti-NMDAR1 antibodies [4]. Moreover, all six of the sections of metastatic and recurrent disease stained strongly positive for expression of *NMDAR1*. Importantly, when the NCI-H345 and NCI-H82 cells were cultured *in vitro* in the presence of a glycine_B site antagonist (L-701,324), the cell viabilities of the cultures increased at 48 h to 140% and 120% of the control values, respectively. These data suggest that antagonizing the binding of glycine contained in the culture medium actually promoted growth of these cancer cell lines. This observation was replicated with another glycine_B site antagonist (L-701,252) using the NCI-H345 cell line. The relevance of NMDAR1 receptors to tumor cell proliferation was further shown with affinity-purified polyclonal antibodies to NMDAR1, which were able to significantly reduce proliferation ($P < 0.001$) at dilutions of 1:10 and 1:50 [4]. These data support the accessibility of the receptor protein on the cell surface. Tumor xenografts of H345 cells were established in nu/nu mice by injecting $\sim 2 \times 10^7$ cells subcutaneously along the flank in 200 μ L of complete medium [4]. Using these tissue xenografts in nu/nu mice, MK-801 (dizocilpine) was shown to have significant effects on slowing tumor growth. Importantly, MK-801 is a non-competitive NMDA receptor antagonist that is an open-channel blocker that binds to a hydrophobic domain within the receptor's ion channel itself; it is only active upon channel opening. Similarly, two breast cancer cell lines (MCF-7 and SKBR3) and eight of 10 breast cancer tumor sections showed evidence of expression of NMDA receptors. [5] Moreover, the NMDA receptor was implicated in regulation of breast cancer cell proliferation because memantine and MK-801 inhibited proliferation and decreased viability of the MC-7 and SKBR3 breast cancer cell lines, and MK-801 inhibited tumor growth of MC-7 tumor xenografts in nu/nu mice [5].

Immunostaining with a rabbit polyclonal antibody that is selective for several splice variants of the NR1 subunit of the NMDA receptor (i.e., NR1-1a, NR1-1b, NR1-2a and NR1-2b) showed that this receptor subunit is expressed in tissue sections of prostate, breast and colon cancer [21]. Expression was also detected in normal prostate and breast sections, but this expression was less intense and observed less frequently in the normal tissues than in the cancer specimens; however, NR1 expression was not observed in stromal or epithelial cells of the eight normal colon specimens. There was also a suggestion that a positive association existed between the staging of prostate cancer specimens and intensity of stromal immunohistochemical staining of the NMDA receptor [21]. Moreover, using a competitive glutamate agonist and non-competitive NMDA receptor antagonist (i.e., memantine), the NMDA receptor was shown to influence rates of proliferation of several cell lines derived from prostate, breast and colon cancers [21].

2. Conclusions

There is no a priori way to predict effects of drugs targeting the open-channel hydrophobic domain to which ketamine, memantine and MK-801 (dizocilpine) bind, the glutamate agonist site, and the glycine_B site on proliferation and invasiveness of NMDA receptor-expressing tumor cells. The effects, if any, on proliferation and invasiveness will likely be determined by the unique combination of receptor subunits and splice variants and phosphorylation state of the NMDA receptor expressed by specific types of tumor tissue. Ultimately, an inventory of the effects of selective NMDA receptor ligands on specific tumor types must be compiled that may guide translational clinical trials.

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Fig. 1.

Regulation of mTOR signaling activity through NMDA receptor activation. The figure depicts NMDA receptor activation leading to both diminished transport of arginine via cationic amino acid transporters (CAT) into the cell, and shortening of the duration of signaling by the phosphorylated form of extracellular signal-regulated kinase (ERK) 1/2.