



2016

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Recommended Citation

Vysotski, S., Winzelberg, R., & Babayeva, M. (2016). Overcoming cancer multidrug resistance through inhibition of microparticles. *GSTF International Journal of Advances in Medical Research*, 1(4).

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Overcoming cancer multidrug resistance through inhibition of microparticles

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Abstract- One of the main obstacles to success of chemotherapy agents is the development of cancer resistance. Cancer multidrug resistance (MDR) is thought to arise from over-expression of efflux transporters on cancer cells' plasma membranes. Recently, microparticles (MP) were found to play a major role in mediating the resistance to antineoplastic agents. Microparticles can confer MDR phenotype to cancer cells through 3 complimentary pathways: 1) Intercellular transfer of P-gp and MRP1; 2) Intercellular transfer of regulatory nucleic acids that ensure acquisition of MDR phenotype; and 3) Internal sequestration of anticancer drugs to reduce the amount of free active drug. Compounds that inhibit MP formation that are currently under investigation include calpain inhibitors, RhoA inhibitors, ROCK inhibitors, calcium channel blockers, pantethine, glutaminase inhibitors, some anti-platelet drugs and some lipid-lowering agents. This area of research requires further development to select, improve and test those compounds that show the most promise in providing safe and effective treatment against MDR.

Keywords: cancer, multidrug resistance, chemotherapy, microparticles

I. INTRODUCTION

Cancer kills millions of people worldwide every year. According to World Health Organization, cancer was a cause of death for 8.2 million people in 2012. Annual cancer rates are rising every year – from 14 million in 2012 to an estimated 22 million in 2030 [1].

One of the main obstacles to success of chemotherapy agents is the development of cancer resistance [2]. There are many mechanisms that contribute to cancer resistance, including mutated expression of drug targets [3], alterations in apoptosis and repair mechanisms [4], reduced drug uptake, increased drug efflux and enzymatic inactivation of drugs [5]. Furthermore, many cancer cells display resistance not only to a single chemotherapeutic agent, but also to a range of structurally unrelated compounds [6]. The phenomenon of resistance to several classes of unrelated agents is termed Multi-Drug Resistance (MDR). MDR is believed to be a contributing factor in 90% of treatment failures in patients with metastatic cancer [7].

Cancer multi-drug resistance is thought to arise from over-expression of efflux transporters on cancer cells'

plasma membranes [8]. When a cancer cell has a large number of efflux transporters on its surface, chemotherapeutic agents get pumped out of the cell before they can harm the cell [9]. Thus, the malignant cells are rendered resistant to chemotherapeutic agents. Most studied efflux transporters essential in the resistance mechanism are those of ABC protein super family: P-glycoprotein (P-gp) and the Multidrug Resistance- Associated Protein 1 (MRP-1) [10].

P-gp is a 170-kDa phosphoglycoprotein which derives energy from hydrolysis of an ATP molecule in order to efflux chemical compounds from the inside of a cell [11]. Its physiological function is to protect both individual cells and the organism as a whole from toxic elements [12, 13]. P-gp substrates are usually hydrophobic organic compounds of large molecular weight (>400g/mol) that carry a positive charge at regular human blood pH [14]. Typical anticancer drugs that are P-gp substrates are anthracyclines, vinca alkaloids and taxanes [15].

MRP1 is a 190 kDa transporter protein that is very similar to P-gp in its function. Even though there is a significant overlap in MRP1 and P-gp substrates, MRP1 is also able to efflux many other kinds of substrates including hydrophilic compounds, glutathione, glucuronide conjugates organic anions and heavy metals [16]. Therefore, MRP1 confers resistance to a broad range of other antineoplastic agents such as methotrexate, etoposide, irinotecan, mitoxantron, antiandrogens and even tyrosine kinase inhibitors [17, 18].

There are two types of tumor resistance: intrinsic and acquired. Intrinsic resistance is usually seen in tumors arising from organs that naturally have numerous efflux pumps such as the intestines, kidneys, adrenal glands, liver, pancreas, brain and lungs [19, 20]. These types of cancers are usually resistant even to the first round of chemotherapy [21]. However, other cancers were shown to be able to acquire the resistant phenotype after exposure to a single chemotherapeutic agent [22]. Statistically, more than 50% of cancer patients end up with the acquired MDR cancers and experience cancer relapse [23]. Five year survival for patients with ovarian cancer is about 30% despite surgical interventions and potent chemotherapy because of the high incidence of acquired MDR [24].

DOI: 10.5176/2345-7201_1.4.32

Since the discovery of the strong link between MDR and efflux transporters, the main strategy in circumventing MDR was the development of efflux pump inhibitors. However, this method has not yet proven viable in a clinical setting because of dose-limiting toxicities and failure to demonstrate survival advantage. Inhibition of efflux transporters led to greater blood brain barrier permeability and caused severe neurologic side effects [25]. Furthermore, past research involved only either P-gp inhibitors or MRP1 inhibitors. Given the substrate redundancy of P-gp and MRP1 transporters, if one is inhibited, the other one may still confer resistance to cancer cells. Therefore, current drug discovery focuses on identifying and studying compounds that inhibit both P-gp and MRP1 simultaneously. No such compounds have yet been discovered. A potent P-gp and MRP inhibitor, VX-710, showed positive results in vitro [26]. However, during Phase II clinical trial only 7 out of 36 patients treated with VX-710 had even a partial response [27]. Currently researchers are investigating other strategies that would overcome MDR with greater efficacy.

In the past, scientists thought that cancer cells were able to acquire the resistant phenotype and over-express efflux transporters only through various genetic and epigenetic changes [28, 29]. Modulation of P-gp and MRP1 expression was reported to be a consequence of increased mRNA stability, gene transcription and gene amplification [30, 31], as well as upregulation of oncogenes and downregulation of tumor suppressor genes [32]. However, recent research proposed and substantiated the idea of non-genetic intercellular transfer of proteins mediated by so called, microparticles [33].

Microparticles (MPs) are small vesicles that are released from the surface of cells by the process of outward membrane budding [34]. They usually express phosphatidylserine (PS) on their outer layer and are about 0.1 to 1 micrometers in diameter [35]. In the past they were considered insignificant blebs that did not have any important function in the body [36]. However, according to the recent research, MPs play an essential role in many physiological functions including intercellular communication, inflammation, coagulation, vascular homeostasis and oncogenic transformation [37, 38]. MPs levels are elevated in many disease states including atherosclerosis, cerebral malaria, HIV, sepsis, different autoimmune disorders and cancer [39], suggesting their role in pathogenesis and a possible therapeutic target.

MPs act as cellular messengers transferring their content short and long distance to recipient cells. MPs can carry diverse types of cargo including cellular proteins (such as efflux transporters), second messengers, cytokines, integrins, transcription factors, and genetic material from their cells of origin [40].

MPs can confer MDR phenotype to cancer cells through 3 complementary pathways: 1) Intercellular transfer of P-gp and MRP1; 2) Intercellular transfer of regulatory nucleic acids that ensure acquisition of MDR phenotype; and 3) Internal sequestration of anticancer drugs to reduce the amount of free active drug.

Intercellular transfer of P-gp through MPs was first reported by Bebawy et al [41]. They observed drug sensitive leukemia cells (CCRF-CEM) acquired functional P-gp after exposure to MPs shed from drug resistant leukemia cells in as little as 4 hours. Likewise, functional MRP1 was detected after 12 hours of co-culture of MPs and drug-sensitive leukemia cells in vitro [42]. An in vivo experiment done on murine tumor xenograph models (MCF-7) also demonstrated ability of MPs to transfer MDR to recipient cells. P-gp loaded MPs were injected subcutaneously near the tumors. In about 24 hours, P-gp could be detected in the recipient tumor cells, and acquired MDR phenotype remained stable for at least two weeks [43].

Acquisition and incorporation of MDR phenotype is also mediated by MPs transfer of regulatory nucleic acids. Especially of interest are microRNAs (miRNAs). MiRNAs are a class of endogenous single stranded non-coding regulatory RNAs that are typically 19-25 nucleotides in length [44]. They modulate activity of specific mRNA targets and regulate protein synthesis [45]. MiRNAs have been shown to significantly affect cellular mechanisms including proliferation, metabolism, apoptosis and resistance to chemotherapeutic agents [46]. Changes in miRNA expression were linked to drug resistance of some common antineoplastics including topotecan, doxorubicin, cisplatin, and methotrexate [47, 48]. Furthermore, recent studies had identified specific miRNAs directly associated with MDR in cancer. For example, miR-27a and miR-451 expression were shown to activate MDR1/P-gp expression in resistant human ovarian cells [49]. Similarly, overexpression of miR-21 and downregulation of the PDCD4 (tumor suppressor protein) were demonstrated to upregulate the number of P-gp expressing breast cancer cells and induce chemoresistance [50]. In addition, miR-297 was also recently found to play a role in development of MDR by modulating MRP expression in colorectal tumors [51]. These studies suggest that miRNAs transferred by MPs from drug resistant to drug sensitive cells are able to transform transcriptional landscape and ensure acquisition of MDR phenotype in the recipient cells by regulating mRNA expression.

Lastly, MPs are also able to sequester drugs within their intravesicular space, which leads to reduced amount of free flowing drug available for anti-tumor action. After MPs were exposed to daunorubicin and doxorubicin, the remaining free drug concentrations were measured using fluorescence analysis and degrees of sequestration were calculated. For drug sensitive MPs degrees of sequestration were 22 and 38 a.u. for daunorubicin and doxorubicin respectively, and for drug resistant MPs - 5 and 4 a.u., respectively. Furthermore, using imaging techniques authors found that drug resistant MPs carried some P-gp transporters in inside-out orientation on their surface. Thus, P-gp acted as influx pumps and helped MPs sequester antineoplastic agents [52].

Recent studies have demonstrated that MPs are elevated in many cancer types including breast [53], gastric [54] and pancreatic [55]. MPs were found to play a critical role not only in cancer drug resistance but in many other aspects of tumor aggressiveness including development of metastases (by transfer of miRNA and matrix degrading proteinases)

[56], angiogenesis (by the dissemination of VEGF) [57, 58], improved cellular survival (by the removal of cytosolic caspase 3) [59] and avoidance of immune surveillance (via expression of LMP-1 and Fas ligand) [60]. Therefore, a growing body of research is focusing on inhibiting cancer microparticle formation [61].

This review will focus on elucidation of MPs biogenesis and enumeration of novel inhibitors of MPs formation that may become effective treatments against cancer multidrug resistance.

II. FORMATION OF MICROPARTICLES

Microparticles are produced by an unusual mechanism that does not require the help of endoplasmic reticulum and Golgi apparatus [62]. Currently, it is thought that microparticles are released from a cell upon cellular activation or apoptosis after disruption of phospholipid asymmetry when PS (that is usually found on the surface of MPs) is redistributed from the inner leaflet of the plasma membrane to the outer leaflet [63]. Cellular activation-induced MP release is associated with the activity of calpains [64], while apoptosis-induced MP formation is regulated by the Rho family of small GTPases [65].

Calpains are calcium-activated cysteine proteinases that are involved in proteolysis, cytoskeletal remodeling, cell motility and apoptosis [66]. There are currently 14 known human calpain isoform genes [67]. The two well-studied members are mu-calpain and m-calpain which differ in their catalytic subunit called calpain-1 and calpain-2, respectively [68]. When a cell is activated, intracellular calcium concentration rises and activates calpain, which hydrolyses the actin binding proteins and disrupts the cytoskeleton immediately under the phospholipid bilayer. These structural changes facilitate microparticle membrane budding [69].

The Rho family of small GTPases, including RhoA, Rac and Cdc42, regulates actin cytoskeleton organization and dynamics [70]. These molecules play a significant role in formation of stress fibers and their signaling pathways affect gene expression and cell survival [71]. Recently, they were also shown to be key regulators of microparticle formation and shedding [72]. RhoA, Rac and Cdc42 are mutated or overexpressed in many kinds of resistant cancers, suggesting their involvement in MDR [73]. The downstream signaling pathway of Rho A that induces MP formation includes Rho-associated coiled-coil containing protein kinase (ROCK), LIM kinase (LIMK) and Cofilin [74]. During apoptosis when cytoskeletal rearrangements occur, activated caspases (enzymes associated with apoptosis) cleave ROCK which fuels cellular transformation and production of MPs [74].

III. INHIBITION OF MICROPARTICLES FORMATION

A. *Is it safe to inhibit microparticles?*

Currently, not everything is known about MP functions in the body, therefore it is difficult to assess safety of microparticle inhibitors in clinical studies. There is a very rare autosomal human disease, called Scott Syndrome, which is characterized by lack of MP formation and impaired thrombin generation resulting in severe bleeding. However, bleeding was not reported to be a side effect in animal models that were given microparticle inhibitors [75]. Moreover, MPs were shown to induce generation of both thrombin [76] and plasmin [77]. Therefore, it is not clear if inhibition of MPs leads to coagulation imbalances or not. In any way, all MP inhibitors available to date do not suppress MP formation completely; and therefore, should not cause serious side effects.

Since MPs play a central role in MDR development and in other manifestations of cancer aggressiveness, much research has been done to identify compounds that may inhibit or modulate MP biogenesis or release from tumor cells. These include calpain inhibitors, RhoA inhibitors, ROCK inhibitors, calcium channel blockers, pantethine, glutaminase inhibitors, some anti-platelet drugs and some lipid-lowering agents.

B. *Calpain Inhibitors*

Calpains are required for MP formation. Increased expression of calpain was observed in several cancer types including schwannomas, meningiomas, renal cell carcinomas and colorectal adenocarcinomas [78, 79, 80]. It has been speculated that inhibiting calpain may prevent microparticle release into the bloodstream and reduce the incidence of acquired MDR. There are several studies showing that calpain inhibitors can decrease the amount of circulating MPs and increase sensitivity of different tumors to multiple structurally unrelated anti-cancer drugs.

Calpastatin is an endogenous inhibitor of calpains [81]. Following calcium influx, calpastatin is released into the cytosol and reversibly inhibits up to four molecules of calpain at once by blocking calpain's active sites [82]. Calpastatin serves as a structural and functional template in the development of novel calpain inhibitors. Most calpain inhibitors available to date target the thiol- containing active site of the calpain. They display limited selectivity to calpains and are often vulnerable to rapid degradation by proteinases *in vivo* [83]. Structure-activity relationship (SAR) studies have fixed pharmacokinetic properties of calpain inhibitors but they were not successful in refining their selectivity [84]. Other calpain inhibitors that target calpain's allosteric site have demonstrated higher selectivity to calpain and are currently under investigation [85].

One calpain inhibitor, MDL-28170, has been shown to significantly reduce MP release from activated platelets [86]. Similarly, another calpain inhibitor, Calpeptin, has been shown to reduce the formation of MPs from activated platelets by about 70% [87]. Moreover, these two calpain inhibitors, Calpeptin and MDL28170, were shown to

increase sensitivity of HER2 positive breast cancer cells to Trastuzumab, a HER2 monoclonal antibody. Skbr3 cells were spread on fibronectin in the presence of Trastuzumab alone as a control or along with each calpain inhibitor. Inclusion of each inhibitor increased cells sensitivity to Trastuzumab by more than 15% ($P < 0.01$) [88].

In another experiment on human melanoma cells allosteric calpain inhibitor, PD150606, combined with a proteasome inhibitor, had significantly reduced viability of cisplatin resistant tumor cells [89]. Furthermore, effect of calpain inhibitors, PD-150606 and ALLM, were studied in drug resistant human breast adenocarcinoma cells. Both inhibitors caused about 20% drop in MP production. Interestingly, PD-150606 worked only on activated cells, whereas ALLM showed an inhibitory effect on both stimulated and unstimulated cancer cells [90].

C. *Inhibitors of Rho-A, Rac, Cdc42 and their downstream effectors*

Rho-A, Rac, Cdc42 and their downstream effectors (LIMK and ROCK) are also essential players in MP biogenesis. Blocking or limiting their function prevents production of MPs and reduces cancer resistance and aggressiveness. Knockout of Rho-A expression using adenovirus-mediated RNA interference inhibited microparticle biogenesis in cervical carcinoma HeLa cells [72]. Similar experiments in lung [91], colorectal [92] and ovarian [93] cancer cells showed that Rho-A knockout decreased proliferation, migration and metastasis of cancer cells, all functions that are associated with MPs. Likewise, AZA1 which inhibits both Rac1 and Cdc42 but not RhoA was found to suppress prostate cell migration and growth [94].

A recent study on breast cancer cell lines revealed that microparticle-mediated acquisition of MDR is closely linked to enhanced metastatic capacity of the recipient cancer cells. As shown in the experiment, when highly metastatic, drug resistant cells were co-cultured with lowly metastatic drug-sensitive cells, the latter ones acquired MDR and an increased metastatic capacity [95]. Putting these studies together reveals that MPs are the source of both metastasis and cancer resistance; and therefore, these compounds that suppress metastasis are possibly also suppressing microparticles and MDR.

Inhibitions of RhoA down-stream effectors, LIMK and ROCK, were also successful in reducing MP production. There is only one study showing that blockage of LIMK expression by LIMK si-RNA inhibits microparticle formation [72]. However, ROCK inhibitors have been extensively studied for more than a decade. They have been proven effective treatments for multiple disease states such as glaucoma [96], ocular hypertension [97], erectile dysfunction [98] and advanced solid tumors [99]. In cancer, ROCK inhibitors were found to suppress tumor invasion, metastasis and MDR [100].

Inhibition of ROCK with Y-27632 compound reduced MP formation in human breast cancer cells by 25% [90]. Another study showed that Y-27632 almost completely inhibited MP formation in various cancerous cell lines, including HeLa cervical cancer cells, MDAMB231 breast cancer cells, and U87 brain tumor cells [101].

Fasudil, initially approved in Japan for treatment of cerebral vasospasms and pulmonary hypertension [102], is the the only clinically available ROCK inhibitor. It has been shown to suppress cancer migration, metastasis [103] and angiogenesis [104]. Moreover, fasudil and another Rho/ROCK inhibitor Y27632 were proven to enhance efficacy of cisplatin. Treatment with cisplatin at 100 microM together with fasudil or Y-27632 showed a synergistic growth inhibitory effect in the cisplatin-resistant cell line. On the other hand, in a cisplatin-sensitive cell line, cisplatin in combination with ROCK inhibitors had similar effects as cisplatin alone. An explanation for the difference in response lies in the understanding of microparticle-mediated drug resistance [105]. ROCK inhibitors increase cisplatin efficacy in cisplatin resistant cell lines because they inhibit microparticle formation, suppressing the transfer of drug resistance and malignant miRNAs between the cells.

One of the newest ROCK inhibitors with improved selectivity and potency, AT13148, which has shown promising results in animal studies, has recently entered Phase I clinical trial for advanced solid tumors [99].

D. *Calcium Channel Blockers (CCBs)*

Increase in intracellular calcium concentration initiates calpain activity and results in MPs formation. Therefore, it has been hypothesized that CCBs are able to decrease amount of MPs in body circulation.

In one experiment, diabetes patients were given benidipine, a dihydropyridine CCB, for 6 months. At the end of therapy, their MP levels were found to be significantly lower than in the beginning [106, 75]. Likewise, in another study, the CCB nifedipine was shown to reduce platelet MPs by about 50 % in patients with transient ischemic attacks [107, 75].

However, in an in-vitro experiment, verapamil, a non-dihydropyridine CCB, did not reduce the number of MPs released from drug-resistant breast cancer cells. On the contrary, verapamil showed a significant increase in MP count relative to the control (by about 45%) [90].

There seems to be a controversy regarding the effect of CCBs on microparticle formation. Perhaps, there is an unknown mechanism that differentiates between dihydropyridines and non-dihydropyridines influence on MPs. Additionally, verapamil was already studied in clinical trials as a P-gp inhibitor and failed to slow the progression of cancer or decrease mortality rates because doses high enough to possibly convey a survival advantage caused intolerable cardiac side effects [108]. Further research is needed to identify those CCBs that can effectively decrease MP levels and not cause cardiac or other complications.

E. Pantethine

Pantethine is a dimer of a pantothenic acid linked by a disulfide cystamine. It has been shown to inhibit the early step of inflammation-coagulation cascade by blocking translocation of phosphatidylserine (PS) [109]. Since movement of PS is important in MP biogenesis, pantethine was studied and was found to decrease MPs both in vitro and in vivo.

After incubation of 1 mM of pantethine with mouse brain endothelial cells, the concentration of MPs was decreased by 51% [110, 75]. A similar experiment with pantethine and human umbilical vein endothelial cells showed MP production reduced by 24% compared to controls. In vivo, malaria-infected mice that were treated with 30mg injections of pantethine for 7 days had significantly lower levels of circulating MPs (by about 50%) compared to control mice that were also infected with malaria but were not treated with pantethine. Interestingly, pantethine did not reduce MP levels in mice not infected with malaria suggesting that pantethine acts selectively on disease-promoting MPs and does not have a negative influence on normal function of MPs in the body [39]. The effect of pantethine on MP formation was also recently studied in tumor cells. Pantethine was incubated with activated drug resistant human breast adenocarcinoma cells for 25 hours, and MP release was quantified by flow cytometry. Pantethine reduced MP formation by 24% relative to control [90].

F. Anti-platelet drugs – Ticlopidine and Clopidogrel

Ticlopidine and clopidogrel are anti-platelet agents used for prevention of thrombosis after a heart attack, stroke, stent placement or other similar conditions. These disease states are associated with high MP levels [111]. Ticlopidine (200mg/day) was shown to reduce MP levels in diabetic patients by 20 to 30%. Nevertheless, even after use of ticlopidine the numbers of MPs were still elevated compared to healthy individuals [111, 75].

The effect of clopidogrel on MP formation was assessed in 26 subjects with stable coronary artery disease. Amount of circulating MPs was inversely correlated with clopidogrel C_{max} and AUC [112]. In addition, in another recent study clopidogrel was found to decrease accumulation of MPs at the site of thrombosis and reduce tumor growth and metastasis in mice with pancreatic cancer [113].

G. Lipid-lowering agents – Statins and EPA/DHA

Statins are drugs of choice for prevention of cardiovascular events. Statins inhibit cholesterol biosynthesis in the liver, and they also have many pleiotropic effects on vascular function including anti-inflammatory and anti-thrombotic effects [114]. Recently, rosuvastatin was reported to influence the number of circulating microparticles. One week after rosuvastatin discontinuation, microparticle levels

significantly increased suggesting its role in suppression of microparticle formation [115]. However, evidence regarding the effect of other statins on MP production is mixed. Atorvastatin decreased platelet derived MPs but increased endothelial MPs [116], while simvastatin had no effect on any microparticles in one study [117], but was found to increase endothelial MPs in another study [118].

EPA/DHA is also used as a lipid-lowering agent and prophylaxis against cardiovascular events. In a 12-week study, EPA/DHA daily use was associated with significantly reduced levels of platelet-derived MPs [119]. Furthermore, when EPA was combined with pitavastatin for a 6 month period in diabetic patients; reduction in platelet-derived MPs was significantly greater than EPA alone (50% vs 20%) [120].

H. Glutaminase inhibitors - BPTES and 968 compounds

Metabolism in cancer cells is slightly different from metabolism in healthy human cells. Healthy human cells usually convert pyruvate into citrate in mitochondria to make ATP. Cancer cells, on the other hand, primarily convert pyruvate into lactic acid, and increase glutamine metabolism to produce alpha-ketoglutarate for entrance into citric acid cycle [121].

Inhibition of glutaminase, an enzyme that catalyzes glutamine transformation into glutamate, was found to inhibit microparticle formation. In an experiment showing that glutaminase activity is linked with microparticle biogenesis, MDAMB23 breast cancer cells were treated with glutaminase allosteric inhibitors, BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethylsulfide) and 968 (bromo- dibenzophenathridine). After a two day period, immunofluorescence analysis showed that untreated cells had 5 times more MP budding than BPTES and 968 treated cells [72]. Thus, BPTES and 968 compounds warrant further research as potential clinically useful inhibitors of microparticle formation.

IV. CONCLUSION

Overcoming cancer MDR is not an easy task. Microparticle's ability to confer MDR by sequestering chemotherapeutic agents and transferring P-gp, MRP1 and miRNA from one cell to another make MPs an excellent target for circumvention of acquired cancer resistance. Many compounds that inhibit MP formation have been identified and are currently under investigation. This area of research requires further development to select, improve and test those compounds that show the most promise in providing safe and effective treatment against MDR.

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