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Liposomal prodigiosin and plasmid encoding serial GCA nucleotides reduce inflammation in microglial and astrocyte cells by ATM/ATR signaling

Mohsen Rashidi[a], Ali Jebali[b,c,*]

[a] Department of Physiology and Pharmacology, Faculty of medicine, Mazandaran University of Medical Science, Sari, Iran
[b] Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[c] Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Ashkezar, Yazd, Iran

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ABSTRACT

The aim of this study was to use liposomal structure consisting prodigiosin and plasmid encoding serial GCA nucleotides (LP/pSGCAN) to reduce inflammation in microglial cells (MGCs) and astrocyte cells (ACCs) by ATM/ATR signaling. Here, it was shown that LP/pSGCAN decreased cell viability and total RNA level. Importantly, LP/pSGCAN had more effect on ACCs than MGCs ($P < 0.05$). Moreover, increase of apoptosis was seen with increase of concentration. The expression of *IL-1* and *IL-6* were decreased and the expression of *ATM* and *ATR* were increased in treated MGCs and ACCs, which showed LP/pSGCAN could inhibit inflammation by activation of ATM/ATR pathway.

1. Introduction

Brain inflammation disease is a condition that the brain and/or spinal cord become inflamed. It leads to irritation and swelling of brain tissue or blood vessels. All inflammatory brain diseases are due to primary processes in which inflammation occurs without a normal trigger. This can lead to brain damage over the long term. Demyelinating conditions, such as multiple sclerosis and acute disseminated encephalomyelitis are often the result of inflammation in the brain (Kim and Joh, 2006).

Ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins are key regulators of the DNA damage response, and maintain genome integrity in eukaryotic cells. *ATM* and *ATR* are expressed in most tissues. Savitsky and co-workers first described *ATM* as the gene responsible for ataxia telangiectasia, whereas *ATR* was first identified and cloned from human T cells. *ATM* and *ATR* belong to the class-IV phosphoinositide 3-kinase (PI3K)-related kinase (PIKK) family, along with mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase (DNA-PK) (Abraham, 2001).

Ribosomal ribonucleic acid (rRNA) is the important part of ribosome. It is essential for protein synthesis in all living cells. rRNAs have a large subunit (LSU) and a small subunit (SSU). LSU rRNA catalyzes peptide bonds, and SSU acts as a carrier (Thomson et al., 2013). As known, rRNA sequences are widely used for evolutionary relationships among organisms. In the most eukaryotes, 18S rRNA is in SSU, and LSU has three rRNA, including 5S, 5.8S, and 28S. The site of ribosomal subunit assembly and rRNA synthesis is nucleolus (Olson and Dundr, 2005). Ribosome has a dynamic structure and its different parts exchange with the nucleoplasm. Ribosome biogenesis is very fine-tuned in response to cellular stress, cell cycle, and cell growth. As an important phenomenon, nucleolus disassembles at the onset of mitosis and reassembles during telophase. This pattern is also seen for rRNA synthesis during prophase and telophase (Dundr et al., 2000). Interestingly, nucleolus also unravels in response to ribosome biogenesis inhibitors, e.g. 5-FU, Actinomycin D, CX-5461, BMH-21, and DRB (Rubbi and Milner, 2003). Sensing cellular stress and aging control are two example of non-conventional roles of ribosomes (Olson et al., 2002; Olson, 2004).

The aim of this study was to use liposomal prodigiosin and plasmid encoding serial GCA nucleotides (LP/pSGCAN) maybe as an rRNA synthesis inhibitor to reduce inflammation in microglial cells (MGCs) and astrocyte cells (ACCs) by means of ATM/ATR signaling.

2. Materials and methods

2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Annexin V, Lipofectamine, prodigiosin, PVDF membrane and all primary antibodies were purchased from Sigma-Aldrich Chemical Co, (St Louis, MO, USA). All primers were sourced from Takapoo Zist

* Corresponding author at: Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd 8916733754, Iran.
E-mail address: alijebal2011@gmail.com (A. Jebali).

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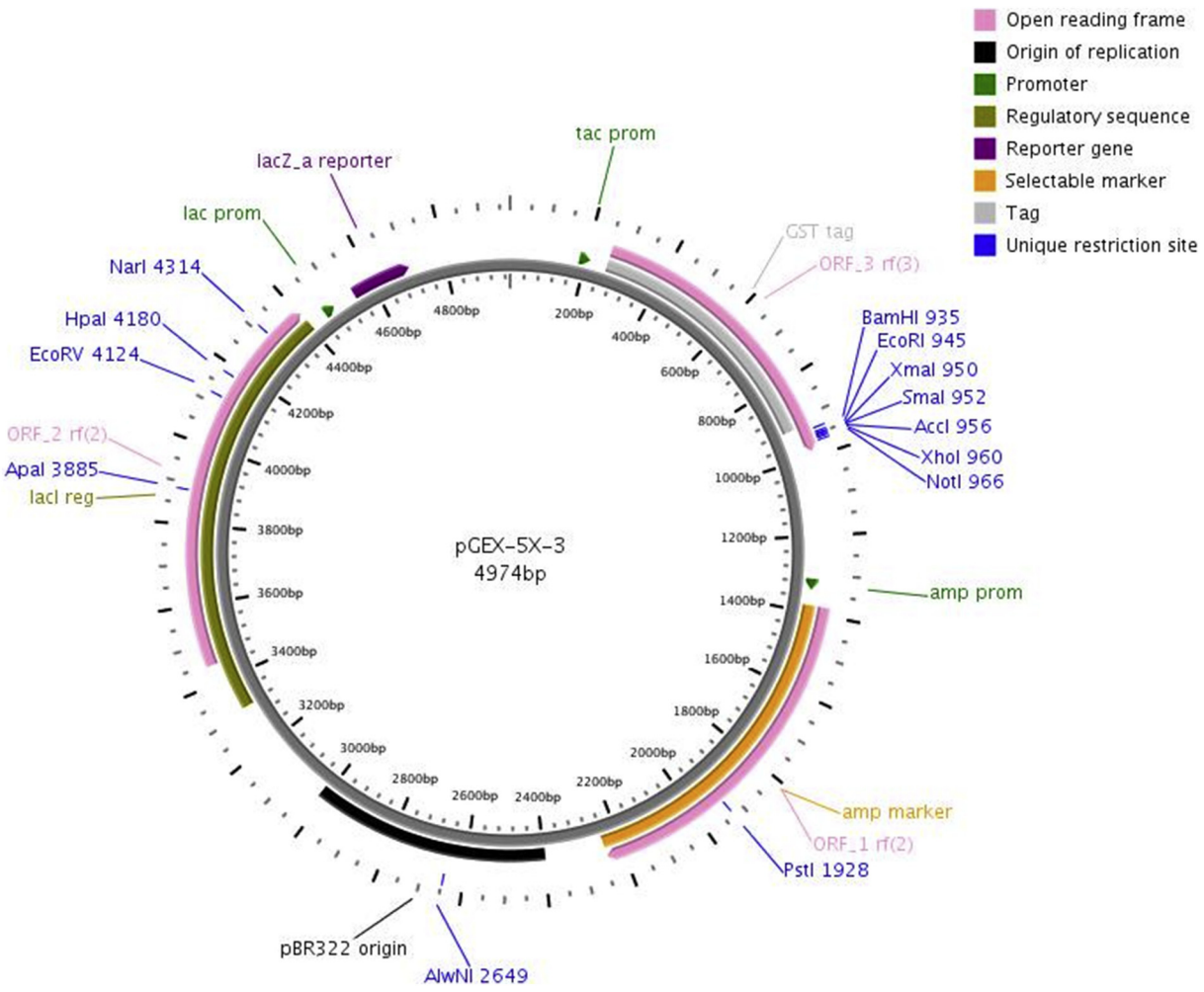

Fig. 1. The schematic presentation of pGEX-5×-3 plasmid, which used in this study.

Company, Iran. RNA extraction buffer (RNX-Plus) and cDNA synthesis kits were purchased from Sinaclon Company, Iran. The real-time Mastermix was provided by Applied Biosystems Company, USA.

2.2. Construction of LP/pSGCAN

Serial GCA nucleotide, 5́-(CGACGACGACGACGACGACGACGACGA CGA)-3́, was synthesized by Takapozyst Company, Iran. Note, the restriction enzyme site of *Bam*HI and *Eco*RI was considered at 3́ and 5́, respectively. Then, both vector (pGEX-5×-3) (Fig. 1) and PCR product were cut by *Bam*HI and *Eco*RI. This construct was built, amplified, and approved by Takara Bio Inc. (Shiga, Japan). To synthesize liposomal form, one mL of prodigiosin (100 mg/mL), one mL of Lipofectamine (1000 nM), and one mL of plasmid encoding serial GCA nucleotides (100 pM) were mixed.

2.3. Cell viability and apoptosis tests

First, MGCs and ACCs (3×10^4) were exposed to LP/pSGCAN (0–2000 nM%), and incubated for 24 h at 37 °C. Finally, cell viability was measured by MTT assay. Annexin V was used for measuring apoptosis. After 24 h, cells were harvested, washed in PBS and suspended in Annexin V binding buffer. Finally, cells were analyzed on flow cytometer.

2.4. Western blot

First, MGCs and ACCs (3×10^4) were exposed to LP/pSGCAN, and then cell lysate was fractionated on SDS-PAGE and transferred to PVDF membrane. Next, PVDF membrane was first blocked with 5% milk and then incubated with primary antibody, including *ATM* and *ATR*.

2.5. Gene expression for ATM/ATR pathway and inflammation

After exposure, total RNA was extracted by RNX- Plus buffer, and then cDNA was synthesized. In the next step, Real-time PCR was performed using SYBR Green Mastermix. Finally, the CT of each gene was read, and its relative expression was measured by ΔΔct method. Primers are listed in Table 1.

Table 1
The sequence of all primers which used in this study.

IL-1	Forward	5́- CAGCCCTTTTGTTGAGCCAG -3́
	Reverse	5́- AGAACCAAAATGTGGCCTGGT -3́
IL-6	Forward	5́- TCAATATTAGAGTCTCAACCCCCAA-3́
	Reverse	5́- CAGGGAGAAGGCAACTGGAC-3́
ATR	Forward	5′- GCGCCACTGAATGAAACTGG −3′
	Reverse	5′- AGGACTACAGCCCATATCAAGC -3′
ATM	Forward	5′- TTTTGTGCCCTTGCAGATTGAT −3′
	Reverse	5′- GGAGCAGGATCCAAATCCCC -3′
GAPDH	Forward	5′ACTTTGGTATCGTGGAAGGAC-3′
	Reverse	5′-CAGTAGAGGCAGGGATGATG-3′

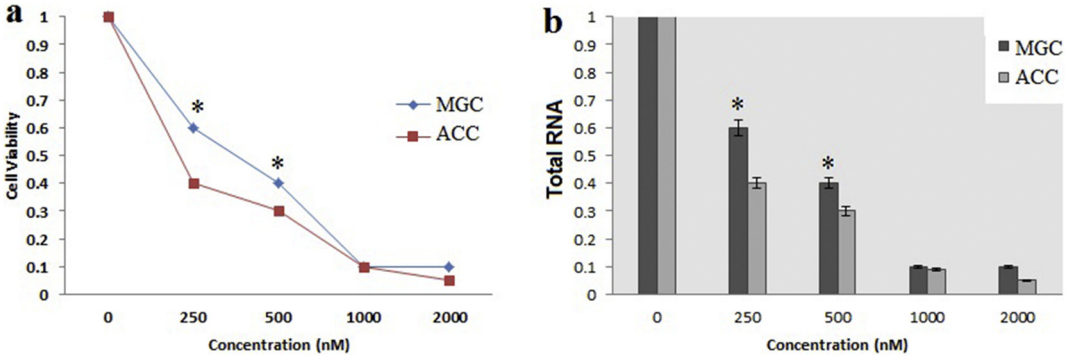

Fig. 2. The cell viability (a) and total RNA level (b) after incubation of MGCs and ACCs with LP/pSGCAN for 1 day. *P < 0.05 compared with ACCs, $n = 15$.

2.6. Statistical analysis

ANOVA was used to identify the possible difference among different treatment groups. Once the difference is confirmed, Student's *t*-test was applied to calculate the significance in the difference between two treatment groups (P values). P-Values < .05 were considered statistically significant.

3. Results

3.1. Cell viability and rRNA levels

Fig. 2 shows cell viability (a) and total RNA level (b) after incubation with LP/pSGCAN for 1 day. As seen, LP/pSGCAN decreased cell viability and total RNA level. The decrease of cell viability and total RNA level was concentration dependent. Importantly, LP/pSGCAN had more effect on ACCs than MGCs (P < 0.05). Fig. 3a shows the quantity of apoptotic cells after incubation with LP/pSGCAN (0–2000 nM) for 1 day. Increase of apoptosis was seen with increase of concentration. Although this pattern was observed in both MGCs and ACCs, treated ACC had more apoptotic cells. Fig. 3b shows the western blot of MGCs and ACCs when they were incubated with LP/pSGCAN (1000 nM) for 1 day. Increased level of ATM/ATR related genes was seen in both MGCs and ACCs.

3.2. ATM/ATR pathway and inflammation gene expression

To find the mechanism of LP/pSGCAN, we checked *IL-1*, *IL-6*, *ATM* and *ATR*. Table 2 shows the expression of *ATM*, *ATR*, *IL-1*, and *IL-6* in MGCs and ACCs when treated with LP/pSGCAN (1000 nM) for 1 day. As seen, the expression of *IL-1* and *IL-6* were decreased in treated MGCs and ACCs. This showed LP/pSGCAN could inhibit inflammation. Also, this study showed the expression of *ATM* and *ATR* were increased in treated MGCs and ACCs. This showed that LP/pSGCAN could activate ATM/ATR pathway.

Table 2
The the expression of *IL-1*, *IL-6*, *ATM*, and *ATR* in MGCs and ACCs when treated with LP/pSGCAN (1000 nM) for 1 day.

	MGCs	ACCs
IL-1	0.84	0.8
IL-6	0.020	0.025
ATM	1.4	1.8
ATR	1.6	2

4. Discussion

Nucleolus is the site of ribosome production. Based on present data, ribosome biogenesis is disrupted by some anticancer drugs, including doxorubicin, camptothecin, CX-5461, BMH-21, DRB, and 5-fluorouracil (Burger et al., 2010). It must be mentioned that although the most of them are not selective to inhibit rRNA synthesis, CX-5461 and BMH-21 are selective (Drygin et al., 2011; Peltonen et al., 2014). When we use a specific molecule as an inhibitor (Jebali et al., 2014), the cell cannot overcome this stress, and it leads to apoptosis. Many proteins are sequestered in the nucleolus and are released in response to specific stimulation, such as rRNA inhibitors (Emmott and Hiscox, 2009; Audas et al., 2012). Wu et al. showed that late S and G2 phase are sensitive to actinomycin D while mitotic cells were resistant (Wu and By-M, 1994). Two phospho-inositide 3-kinase-like protein kinases (PIKKs), including ATM and ATR are important regulators of DNA damage. ATM is primarily activated by DNA double-strand breaks (DSBs), and ATR responds to broad spectrum of DNA damage. It was found that ATR has an important role in stabilizing the genome during DNA replication. The initial step in ATR activation is the recognition of DNA structures that

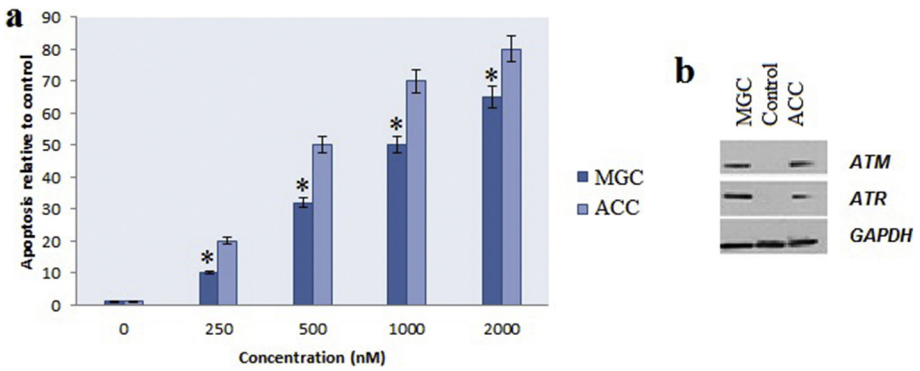

Fig. 3. The quantity of apoptotic cells (a) and western blot (b) after incubation of MGCs and ACCs with LP/pSGCAN for 1 day. *P < 0.05 compared with ACCs, n = 15.

are induced by DNA damage, such as single-stranded DNA (ssDNA) and junctions between ssDNA and double-stranded DNA (dsDNA). An increased amount of ssDNA is generated at DNA replication forks when the coordination between DNA polymerase activity and DNA helicase activity is compromised. In addition, ssDNA gaps are induced by several types of DNA repair (Myers and Cortez, 2006). ssDNA is also present at DSBs that have been trimmed by *exo-* or endonucleases through a process called resection. In all eukaryotes, ssDNA that is induced by DNA damage is first detected by replication protein A (RPA). ATR-interacting protein (ATRIP), which is the regulatory partner of ATR, binds directly to RPA-coated ssDNA (RPA-ssDNA) and thereby enables the ATR-ATRIP complex to localize to sites of DNA damage (Zou and Elledge, 2003). Additional interactions between ATR-ATRIP and RPA, as well as the interactions between ATR-ATRIP and other proteins, might also contribute to the association of ATR-ATRIP with damaged DNA (Kumagai et al., 2006).

In this study, it was shown that LP/pSGCAN decreased cell viability and total RNA level. Importantly, LP/pSGCAN had more effect on ACCs than MGCs ($P < 0.05$). Increase of apoptosis was seen with increase of concentration. The expression of *IL-1* and *IL-6* were decreased in treated MGCs and ACCs. This showed LP/pSGCAN could inhibit inflammation. Also, this study showed the expression of *ATM* and *ATR* were increased in treated MGCs and ACCs. This showed that LP/pSGCAN could activate ATM/ATR pathway.

Trickler et al. examined the interactions of silver nanoparticles (Ag-NPs) with the cerebral microvasculature to identify the involvement of proinflammatory mediators. They showed that Ag-NPs could increase blood-brain barrier permeability. The pro-inflammatory responses in this study demonstrated both Ag-NPs size and time-dependent profiles, with IL-1B preceding both TNF and PGE2 for 25 nm (Trickler et al., 2010). Jain et al. showed that RGD-anchored magnetic liposomes can be used for monocytes/neutrophils-mediated brain targeting. Results suggest that selective uptake of RGD-anchored magnetic liposomes by these cells imparts them magnetic property. In case of negatively charged uncoated magnetic liposomes brain levels of the drug was 5.95-fold compared to free drug and 7.58-fold in comparison to non-magnetic formulation, while for RGD-coated magnetic liposomes this ratio was 9.1-fold compared to free drug solution, 6.62-fold compared to non-magnetic RGD-coated liposomes and 1.5-fold when compared to uncoated magnetic liposomes (Jain et al., 2003). Qin et al. demonstrated that surface modification of RGD-Liposomes can be used for selective drug delivery to Monocytes/Neutrophils in brain. The body distribution results showed that RGD-liposomes could be directed to the target site, i.e. the brain, by cell selectivity in case of an inflammatory response. For RGD coated liposomes, the concentration of FA in brain was 6-fold higher than that of FA solution and 3-fold higher than that of uncoated liposomes (Qin et al., 2007). Gaillard et al. nnhanced brain delivery of liposomal methylprednisolone in a model of neuroinflammation. Free methylprednisolone and non-targeted pegylated (PEG) liposomal methylprednisolone served as control treatments. When treatment was initiated at disease onset, free methylprednisolone showed no effect, while GSH-PEG liposomal methylprednisolone significantly reduced the clinical signs to 42 ± 6.4% of saline control (Gaillard et al., 2012).

5. Conclusion

Taken together, it was found that LP/pSGCAN decreased cell viability and total RNA level. Importantly, LP/pSGCAN had more effect on ACCs than MGCs ($P < 0.05$). Increase of apoptosis was seen with increase of concentration. The expression of *IL-1* and *IL-6* were decreased in treated MGCs and ACCs. This showed LP/pSGCAN could inhibit inflammation. Also, this study showed the expression of *ATM* and *ATR* were increased in treated MGCs and ACCs. This showed that LP/pSGCAN could activate ATM/ATR pathway.

Conflict of interest

There is no conflict of interest to declare.

Acknowledgements

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References

Abraham, R.T., 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. 15 (17), 2177–2196.
Audas, T.E., Jacob, M.D., Lee, S., 2012. Immobilization of proteins in the nucleolus by ribosomal intergenic spacer noncoding RNA. Mol. Cell 45 (2), 147–157.
Burger, K., Mühl, B., Harasim, T., Rohrmoser, M., Malamoussi, A., Orban, M., et al., 2010. Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. J. Biol. Chem. 285 (16), 12416–12425.
Drygin, D., Lin, A., Bliesath, J., Ho, C.B., O'Brien, S.E., Proffitt, C., et al., 2011. Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth. Cancer Res. 71 (4), 1418–1430.
Dundr, M., Misteli, T., Olson, M.O., 2000. The dynamics of postmitotic reassembly of the nucleolus. J. Cell Biol. 150 (3), 433–446.
Emmott, E., Hiscox, J.A., 2009. Nucleolar targeting: the hub of the matter. EMBO Rep. 10 (3), 231–238.
Gaillard, P.J., Appeldoorn, C.C., Rip, J., Dorland, R., van der Pol, S.M., Kooij, G., et al., 2012. Enhanced brain delivery of liposomal methylprednisolone improved therapeutic efficacy in a model of neuroinflammation. J. Control. Release 164 (3), 364–369.
Jain, S., Mishra, V., Singh, P., Dubey, P., Saraf, D., Vyas, S., 2003. RGD-anchored magnetic liposomes for monocytes/neutrophils-mediated brain targeting. Int. J. Pharm. 261 (1–2), 43–55.
Jebali, A., Hajjar, F.H.E., Hekmatimoghaddam, S., Kazemi, B., Jesus, M., Rashidi, M., 2014. Triangular gold nanoparticles conjugated with peptide ligands: a new class of inhibitor for Candida albicans secreted aspartyl proteinase. Biochem. Pharmacol. 90 (4), 349–355.
Kim, Y.S., Joh, T.H., 2006. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. Exp. Mol. Med. 38 (4), 333–339.
Kumagai, A., Lee, J., Yoo, H.Y., Dunphy, W.G., 2006. TopBP1 activates the ATR-ATRIP complex. Cell 124 (5), 943–955.
Myers, J.S., Cortez, D., 2006. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. J. Biol. Chem. 281 (14), 9346–9350.
Olson, M., 2004. Sensing cellular stress: another new function for the nucleolus. pp. pe10.
Olson, M., Dundr, M., 2005. The moving parts of the nucleolus. Histochem. Cell Biol. 123 (3), 203–216.
Olson, M.O., Hingorani, K., Szebeni, A., 2002. Conventional and nonconventional roles of the nucleolus. Int. Rev. Cytol. 219, 199–266.
Peltonen, K., Colis, L., Liu, H., Trivedi, R., Moubarek, M.S., Moore, H.M., et al., 2014. A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. Cancer Cell 25 (1), 77–90.
Qin, J., Chen, D., Hu, H., Cui, Q., Qiao, M., Chen, B., 2007. Surface modification of RGD-liposomes for selective drug delivery to monocytes/neutrophils in brain. Chem. Pharm. Bull. 55 (8), 1192–1197.
Rubbi, C.P., Milner, J., 2003. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. EMBO J. 22 (22), 6068–6077.
Thomson, E., Ferreira-Cerca, S., Hurt, E., 2013. Eukaryotic ribosome biogenesis at a glance. J. Cell Sci. 126 (21), 4815–4821.
Trickler, W.J., Lantz, S.M., Murdock, R.C., Schrand, A.M., Robinson, B.L., Newport, G.D., et al., 2010. Silver nanoparticle induced blood-brain barrier inflammation and increased permeability in primary rat brain microvessel endothelial cells. Toxicol. Sci. 118 (1), 160–170.
Wu, M.-H., By-M, Yung, 1994. Cell cycle phase-dependent cytotoxicity of actinomycin D in HeLa cells. Eur. J. Pharmacol. 270 (2–3), 203–212.
Zou, L., Elledge, S.J., 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 300 (5625), 1542–1548.