

Buthionine Sulfoximine Inhibits Cytopathic Effects and Apoptosis Induced by Infection with AIK-HDC Strain of Measles Virus

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ABSTRACT

Background: Measles virus (MV) is a highly contagious agent which causes a major health problem in developing countries. We studied the effect of buthionine sulfoximine (BSO) on the replication of an AIK-HDC strain of MV and its induced apoptosis in Vero cell lines. **Methods:** In this study, toxicity of BSO on Vero cells was investigated first, resulted in determination of sub-lethal or non-toxic concentration zone of BSO for cells. Next, anti-viral effect of BSO at various time limits was evaluated and virus titer was determined at each stage either as 50% tissue culture infective dose (TCID)₅₀ or by plaque assay method. Using specific anti-measles IgG, anti-viral effect of BSO on MV replication cycle was evaluated through indirect immunofluorescence assay, meanwhile presence of viral RNA was investigated by RT-PCR and gel electrophoresis. **Results:** According to the experiments, BSO, at concentration of 50 µM, markedly inhibited the cytopathic effect (CPE) induced by MV. BSO also significantly inhibited apoptosis induced by MV. BSO either influences replication of MV genome, or may inhibit virion formation. **Conclusion:** These results suggest that the inhibition of CPE and apoptosis by BSO induced by MV may be associated with the effect of BSO on viral RNA genome. Therefore, it is suggested that MV infections can induce apoptosis through the activation of a common pathway that can be inhibited by BSO. *Iran. Biomed. J. 11 (4): 229-235, 2007.*

Keywords: Measles virus (MV), AIK-HDC, Buthionine sulfoximine (BSO), Apoptosis

INTRODUCTION

Measles virus (MV) is a member of Morbillivirus of the Paramyxoviridae family. MV is responsible for an acute respiratory disease with high morbidity and over 1,000,000 mortality around the world in a year [1]. In some cases, MV induces an acute disseminated encephalomyelitis and more rarely, measles inclusion body encephalitis or subacute sclerosing panencephalitis. It is now thought that still approximately 30 million individuals are infected with measles and 0.77 million children die of measles or measles-related complications throughout the world every year [2].

Apoptosis is characterized by accompanying morphological changes, such as chromatin condensation, cell shrinkage, nuclear fragmentation, and apoptotic body formation [3]. The biological hallmark of apoptosis is internucleosomal DNA fragmentation [4]. Apoptosis is reportedly induced

by infection with isolates of human adenovirus, Human herpesvirus 1, and MV [5, 6] as well as human coxsackievirus B3 (CB3) [7].

Reactive oxygen species are major factors in the induction of apoptosis which is associated with the activation of caspase and protein kinase signal transduction cascades. Glutathione (GSH) plays an important role in maintaining the cellular reduction-oxidation potential. Buthionine sulfoximine (BSO), a synthetic amino acid, depletes cells of GSH, a metabolite that plays a critical role in protecting cells against oxidative stress and resulting in free radical-induced apoptosis. On the other hand, BSO is an irreversible inhibitor of gamma-glutamylcysteine synthetase [8] that is a key enzyme of GSH biosynthesis, and the agent causes a rapid and remarkable decrease in intracellular GSH. Therefore, BSO has been used as an inducer of oxidative stress in both *in vitro* and *in vivo* studies [9, 10]. With respect to GSH depletion and alteration in cellular redox potential, virus can no longer

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replicate regularly in cell culture. Therefore cytopathic effect (CPE) and subsequent cell apoptosis may be prevented or at least decreased.

Our main objective of this study is to evaluate effect of BSO on cytopathic formation and apoptosis induced by MV. The other objective is to clarify whether the alteration of reduction-oxidation potential affects MV replication and virus-induced cell death.

MATERIALS AND METHODS

Viruses. MV, AIK-HDC strain was obtained from Razi Vaccine and Serum Institute (Karaj, Iran). Before anti-viral testing can be carried out, it was essential to obtain a seed stock of the virus with known infectivity. Virus titer was determined either as 50% tissue culture infective dose (TCID)₅₀ or by plaque assay method.

Cell culture. Vero cells were seeded at a density of 10⁶ cells/ml, containing 5-6 ml of DMEM supplemented with 8-10% fetal bovine serum at 37°C for 2-3 days. The cells were infected with 10 TCID₅₀/cell of MV, washed with PBS to remove free virions. Then, cells were treated with 50, 25, 10 and 5 µM BSO (non-toxic concentrations) in maintenance medium supplemented with 1% fetal bovine serum. Cells were examined daily for the appearance of CPE.

Effect of BSO on non-infected Vero cells. Various concentrations of BSO (200, 100, 50, 25, 10 and 5 µM) were added to monolayers of Vero cells. Cells were incubated at 37°C up to 4 days. The count is then mathematically converted to the number of cells per milliliter. The stained (dead) cells and unstained (living) cells were counted separately to determine the percentage that was viable.

Plaque assay method. CPE Vero cells were seeded at a density of 10⁶ cells/ml in culture flask and infected after 24 hours with respective virus dilutions (10⁻³-10⁻⁸) to each duplicate flask, incubated at 37°C for 1 hour. Sequentially, the virus was removed from the flasks, discarded and replaced quickly with 4-5 ml of diluted agarose to avoid desiccation of monolayer. After 10-20 minutes, for gel hardening, flasks were incubated at 37°C in a humidified incubator for 4-7 days. All flasks were monitored daily until the number of plaques counted did not change for two consecutive days. Then,

bottles were overlaid with 1-2 ml dye solution included a final concentration of 50 µg/ml neutral red and bright plaques in pinky background were counted and virus titer was calculated.

Virucidal effect of BSO on MV. MV with titer of 10⁶ TCID₅₀/ml was exposed to 50 µM of BSO and was incubated in two thermal conditions (4 and 37°C) for 1 h. Control virus was treated similarly but with no BSO. Both viral preparations were titred by TCID₅₀ method.

Effect of BSO on MV replication. To examine the step on which BSO can interfere with MV replication cycle, Vero cells were seeded at a density of 10⁶ cells/ml in 24 wells micro plates. Cells were infected with MV at multiplicity of infection (moi) of 10, inoculated for 60 min and washed with PBS to remove free virions. BSO (50 µM) was added to the infected cells at various time intervals (16, 24, 48, 72 and 96 h after infection) and were incubated in DMEM supplemented with 1% fetal bovine serum. Cultures were assayed for virus infectivity after next 4-5 days.

Indirect immunofluorescence assay. Cover slip cultures of Vero cells in 24 wells micro titer plate were infected with MV at moi of 10 with or without 50 µM BSO and incubated in 37°C after 2-3 days. Coverslips were washed in 2 changes of PBS for 2 min. and fixed in -20°C acetone for 10 min at -20°C. Anti-measles IgG was added to cover slips, incubated in humidified 37°C for 40 min. They were washed in PBS and anti-IgG conjugate (FITC) was added to cover slips, incubated in humidified 37°C for 30 min. Cover slips were washed in PBS and mounted using glycerol buffer.

RT-PCR. Vero cells at first, second and third passages of MV were centrifuged at 10,000 × g for 10 min and the supernatant was used for the detection of MV RNA. Total RNA was extracted from 250 µl of the supernatant using AccuPrep Viral RNA Extraction Kit (Bioneer, Daejeon, Korea). cDNA synthesis was carried out by AccuPower RT/PCR PreMix (Bioneer, Daejeon, Korea). For PCR, 5 µl of the cDNA sample was added to a 45 µl PCR mixture. PCR conditions were as follows: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. After 39 cycles, each sample was analyzed by electrophoresis on a 1.5% agarose gel. DNA was visualized by ethidium bromide staining and UV illumination. The following oligonucleotides which

amplify 292 bp of Nucleoprotein of MV [11] were used: forward 5'-CATTACATCAGGATCCGG-3' and reverse 5'-GTATTGGTCCGCCTCATC-3'.

Detection of apoptosis by agarose gel electrophoresis. Infected Vero cells with or without BSO were collected by centrifugation at $2,000 \times g$ for 10 min, and DNA was prepared with the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The DNA was electrophoresed on 2% agarose gel, and visualized with ethidium bromide. Apoptosis was evaluated based on observation of a ladder of 180 bp DNA fragment unit.

Detection of apoptotic cells. Vero cells were cultured on tissue culture chamber slides under the condition mentioned above. Cells (4×10^3) were maintained in various concentrations of BSO, infected at moi of 10 and harvested at various time points. Apoptotic cells were detected with ApopTag plus Fluorescein *in situ* Apoptosis Detection Kit (Chemi-Con International, CA, U.S.A.), an *in situ* apoptosis detection kit. In brief, cultured cells were incubated with terminal deoxynucleotidyl transferase-and digoxigenin-labelled dUTP. They were then treated with anti-digoxigenin affinity purified sheep polyclonal antibody conjugated to fluorescein. Cells were counterstained with propidium iodide and ratios of apoptosis were estimated by counting 1,000 cells showing fluorescence.

Quantification of GSH content. After inducing apoptosis in Vero cells by MV, infected cells were treated with 50 μ M BSO for 2-3 days. For preparation of cytosolic fraction, $1-5 \times 10^6$ cells were washed once with 5-10 ml of ice cold 1X wash buffer. Culture dishes were placed on ice and were added by 1 ml of prechilled 1X cell lysis buffer. After incubation for 10 min on ice, cells and debris were collected with a rubber policeman. Cell extract was transferred into a micro centrifuge tube and centrifuged at $12000 \times g$ for 10 min, supernatant was collected into a new centrifuge tube and used for GSH assay. GSH content was determined using a GSH Detection Kit (Chemi-Con International, CA, and USA). In brief, to cytosolic fraction we added 90 μ l of lysate and 10 μ l of prepared MCB solution ($C_{10}H_{11}ClN_2O_2$) to a 96-well plate suitable for fluorometer and incubated 1-2 h at room temperature away from light. The fluorescence was read using a 380/460-nm filter set in a fluorometer.

RESULTS

Suppressive effect of BSO on CPE and apoptosis induced by MV. We first examined the effect of BSO on morphological changes in Vero cells; no changes were observed between non-treated and BSO-treated cells using final concentration of 50 μ M BSO. We next compared the morphological changes induced by MV infection between BSO-treated and non-treated Vero cells. CPE was clearly observed in drug-free cells 2 days after infection; while CPE was inhibited in BSO-treated cells (Fig. 1). Cytotoxicity

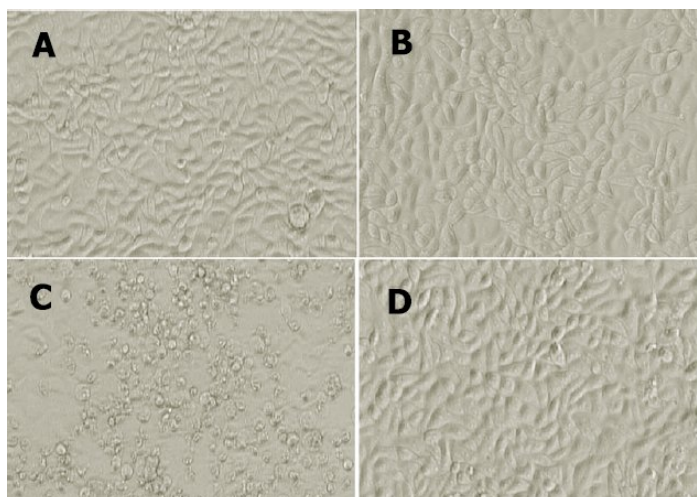


Fig. 1. Inhibition of CPE by BSO induced by measles virus. (A) non-infected Vero cells without BSO; (B) non-infected Vero cells with BSO; (C) measles virus infected Vero cells without BSO and (D) measles virus infected Vero cells with BSO. Magnification is $\times 200$.

was evaluated using the trypan blue staining method and by examining cell morphology by light microscopy. Simply, while BSO had its activity, MV did not show any activity or its activity is remarkably decreased up to a concentration of 50 μ M of BSO. According to the experiments, BSO at a concentration more than 100 μ M have remarkable cell cytotoxicity, but have no adverse effect at a range of 50 μ M and below (Fig. 2).

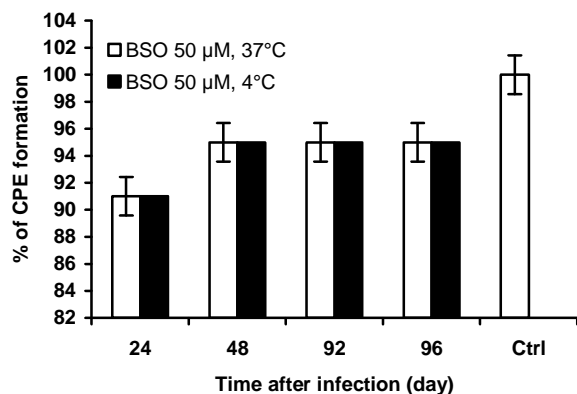


Fig. 2. Virucidal effect of BSO on measles virus. BSO at a concentration of 50 μ M have no effect on virus titer at various time limits and so have no virucidal effect on measles virus.

Anti-viral activity of BSO was examined by a virus yield inhibition assay. Extracellular exposure of MV by BSO indicated that BSO at a concentration of 50 μ M, have no effect on virus titer at various time limits and so have no virucidal effect on MV (Fig. 3). BSO at a concentration of 50 μ M markedly inhibit cytopathic formation induced by MV. This inhibitory effect was considerable such that third passage virus titer almost diminishes (Fig. 4). Based on the results, the best post infecting time to add BSO, determined as 24 h (Fig. 5).

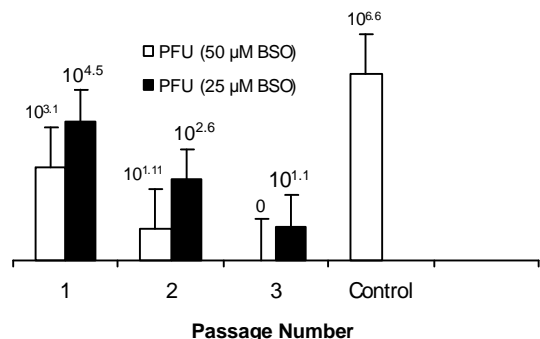


Fig. 3. Virus titer in BSO-treated and non treated infected cells at passage number 1 to 3. The inhibitory effect was considerable at concentration of 50 μ M, at third passage virus titer almost diminishes.

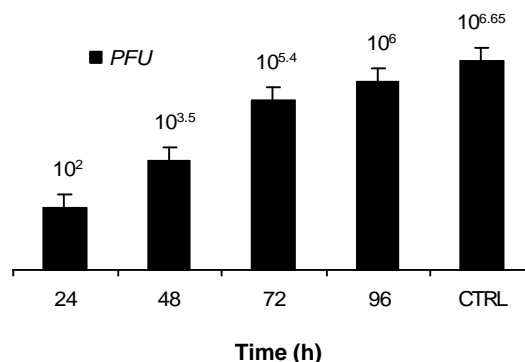


Fig. 4. Virus titer in BSO-treated infected cells at various post infection time points. The best post infecting time to add BSO determined as 24 h.

To evaluate the synthesis of viral proteins, presence of viral antigens in infected cells in presence and absence of BSO was evaluated using indirect immunofluorescence assay. The results showed that amount of viral antigens reduced extensively in BSO-treated cells at passage no. 2.

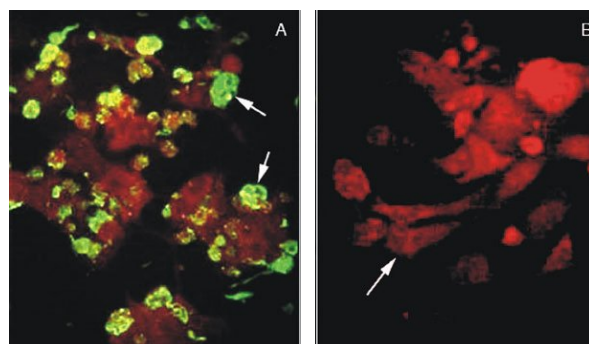


Fig. 5. Detection of measles virus antigens using immunofluorescence assay. (A) Infected Vero cells at 2nd passage treated without BSO; (B) Infected Vero cells at 2nd passage with BSO. The results showed that amount of viral antigens reduced extensively in BSO-treated cells at passage no.2.

These data show that viral protein synthesis in cell cultures had extremely affected by BSO treatment; and that maybe result in decrease in CPE formation due to viral infection in BSO-treated cells (Fig. 6).

To evaluate the inhibitory effect of BSO on apoptosis induced by MV, Vero cells were infected with MV of 10 TCID₅₀. Cells were harvested at the indicated time points and DNA was extracted. The DNA (1 μ g) was electrophoretically analyzed on 1.5% agarose gel. Vero cells were seeded into 3-cm plastic dishes and cultured in a medium with or without 50 μ M BSO. After 2-3 days, the cultures were infected with 10 TCID₅₀ of MV. Following

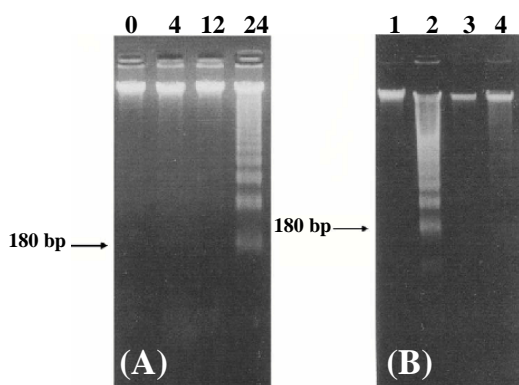


Fig. 6. Inhibition by BSO of apoptosis induced by measles virus. (A) DNA was analyzed on 1.5% agarose gel and apoptosis was detected after 24 h. Based on observation of a 180 bp ladder. Fragmentation unit; (B) lane 1, non-infected Vero cells without BSO; lane 2, measles virus infected Vero cells without BSO; lane 3, measles virus infected Vero cells with BSO; lane 4, non-infected Vero cells with BSO.

culture for 24 h, these cells were harvested, DNA was extracted and electrophoresed on agarose gel. The results showed that in the absence of BSO, cDNA obtained from infected cells was fragmented whereas in presence of BSO this fragmentation was inhibited as illustrated in Figure 7.

To investigate the Effect of BSO on MV replication, Vero cells were treated with BSO and infected with MV as described in Figure 1. After freezing and thawing, cells were centrifuged at $10000 \times g$, and the resulting supernatant was used for RT-PCR. Amplified cDNA was separated on 2% agarose gel. According to these results, the presence of viral RNA genome in BSO-treated and non-treated infected cells at second passage was negative and positive, respectively. Data shows that MV can not regularly replicate at the presence of indicated concentration of BSO as illustrated in Figure 8.

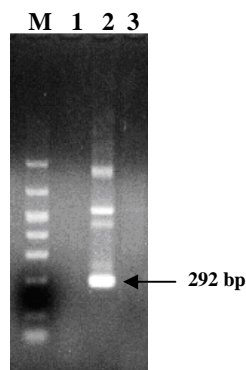


Fig. 7. Effect of BSO on measles virus replication. M, size marker; lane 1, non-infected Vero cells without BSO; lane 2, measles virus infected Vero cells without BSO; lane 3, measles virus infected Vero cells with BSO.

We examined whether a change of reduction-oxidation state might influence CPE induced by MV infection in Vero cells. As indicated, the quantity of intracellular GSH markedly decreased after infection by MV and also treatment with BSO. According to these data, BSO can decrease intracellular level of GSH enormously and then MV can no longer exist or replicate properly in such altered cells. Subsequently, it can not produce CPE as before and apoptotic cells due to virus infection extremely prevented or at least decreased.

Table 1. The effect of BSO on cell growth and toxicity. BSO at a concentration more than 100 μM have remarkable cell cytotoxicity, but have no adverse effect at a range of 50 μM and below.

BSO μM	Viable cells % 1 st day	Viable cells % 2 nd day	Viable cells % 3 rd day	Viable cells % 4 th day
0	98	98	96	95
400	30	25	24	24
300	31	25	24	23
200	35	30	28	28
100	87	84	80	80
50	97	96	95	95

DISCUSSION

Measles is an acute infectious disease which is considered as one of the major cause of child death worldwide. Despite the availability of a safe, effective, and relatively inexpensive vaccine for over 40 years, measles remains the leading cause of child mortality among vaccine-preventable diseases. The WHO estimated that 30-40 million measles cases and 530,000 measles-related deaths occurred worldwide in 2003. There is currently no specific treatment for measles infection. Administration of vitamin A to children with measles has been shown to decrease both the severity of the disease and the case-fatality rate. WHO recommends that vitamin A should be administered to all children with acute measles. Supportive treatment should be provided for a number of measles complications.

These findings suggest that BSO at given concentrations have an inhibitory effect on growth and replication of MV. As mentioned, there is a considerable decrease in virus titer at passage number 2 that almost diminished at passage number 3 and there was no viral antigen at this stage. According to the experiments, BSO, at a concentration of 50 μM , markedly inhibited the CPE induced by MV. BSO also significantly inhibited apoptosis induced by MV. BSO either influences

replication of MV genome, or may inhibit virion formation. These results suggest that the inhibition of CPE and apoptosis by BSO induced by MV may be associated with the effect of BSO on viral RNA genome. Therefore, it is suggested that MV infections can induce apoptosis through the activation of a common pathway that can be inhibited by BSO.

It has been reported that cellular oxidation triggers viral replication such as human immunodeficiency viruses and Influenza A virus [12, 13]. In the present study, we demonstrated that BSO remarkably inhibited CPE induction by MV. Since exogenous GSH reversed the inhibitory effect of BSO on the CPE induced by MV infection like EV9 [14], the inhibitory effect may be attributed to intracellular oxidation caused by BSO. Moreover, the inhibitory effect of BSO may not be cell-specific, because BSO also attenuated the induction of CPE by MV infection in B95-8. The inhibitory effect of BSO was also observed in other viruses like type species of the genus Enterovirus [15], indicating that BSO may inhibit a common apoptotic pathway triggered by enteroviral infection. In this study, it was also showed that BSO influenced replication of MV genome but it may inhibit virion formation in lesser amount. These results suggest that BSO fails to modulate the receptor-mediated cell attachment but may inhibit MV genome replication and maybe virus maturation. It has been reported that apoptosis induced by CB3 is dependent on the stimulation of a mitogen activated protein kinase pathway [15] which is activated under oxidative conditions [16]. Alternatively, poliovirus protease 3C reportedly triggers apoptosis in infected cells via a mechanism that involves caspase activation [17].

It is not clear whether CPE and apoptosis are associated with cellular events accompanying MV infection. Carthy *et al.* [7] reported that benzyloxycarbonyl Val-Ala-Asp-fluoromethylketone (ZVAD-fmk), a caspase inhibitor, did not inhibit a CPE induced by CB3. In addition, Agol *et al.* [18] indicated that pathways leading to CPE and apoptosis induced by isolates of Poliovirus are different. It is possible that the pathway of CPE may be different from that of apoptosis during MV infection. Further study is necessary to clarify the pathway of apoptosis induced by MV infection and the inhibitory mechanism of BSO.

These results may help us to find a parallel treatment to control measles and some diseases that their ethological agents are related to MV; e.g. RSV that causes a serious problem for lesser children.

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REFERENCES

1. Santos, P.R., Borges, M.B.J. and Freire, M.S. (2003) Comparative sequence analysis of the P-, M- and L-coding region of the measles virus CAM-70 live attenuated vaccine strain. *Braz. J. Med. Biol. Res.* 36: 1475-1484.
2. WHO (2002) Global measles mortality reduction and regional elimination, 2000-2001, Parts 1 and 2. *Wkly Epid. Res.* 77: 50-5, 58-61.
3. Arends, M.J., Morris, R.G. and Wyllie, A.H. (1990) Apoptosis: the role of endonuclease. *Am. J. Pathol.* 136: 593-608.
4. Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1981) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251-306.
5. Lisa, M., Esolen, B.J., Ward, T.R. and Griffin, D.E. (1995) Apoptosis as a cause of death in measles-infected cells. *J. Virol.* 69: 3955-3958.
6. Galvan, V. and Roizman, B. (1998) Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type dependent manner. *Proc. Natl. Acad. Sci. USA* 95: 3931-3936.
7. Carthy, C.M., Granville, D.J., Watson, K.A., Anderson, D.R., Wilson, J.E., Yang, D., Hunt, D.W.C. and McManus, B.M. (1998) Caspase activation and specific cleavage of substrates after coxsackievirus B3-induced cytopathic effect in HeLa cells. *J. Virol.* 72: 7669-7675.
8. Hamilton, T.C., Winker, M.A., Louie, K.G., Batist, G., Behrens, B.C., Truto, T., Grotzinger, K.R., Mekoy, W.H., Young, R.C. and Ozols, R.F. (1985) Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell line by buthionine sulfoximine mediated glutathione depletion. *Biochem. Pharmacol.* 34: 2583-2586.
9. Lin, A.M., Chen, C.F. and Ho, L.T. (2002) Neuroprotective effect of intermittent hypoxia on iron-induced oxidative injury in rat brain. *Exp. Neurol.* 176: 328-335.
10. Smith, S.L., Sadler, C.J., Dodd, C.C., Edwards, G., Ward, S.A., Park, B.K. and McLean, W.G. (2001) The role of glutathione in the neurotoxicity of artemisinin derivatives *in vitro*. *Biochem. Pharmacol.* 61: 409-416.
11. Rota, J.S., Heath, J.L. and Rota, P.A. (1996) Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination. *J. Infect. Dis.*

- 173: 32-37.
12. Cai, J., Chen, Y., Seth, S., Furukawa, S., Compans, R.W. and Jones, D.P. (2003) Inhibition of influenza infection by glutathione. *Free Radic. Biol. Med.* 34: 928-936.
 13. Nencioni, L., Iuvara, A., Aquilano, K., Ciriolo, M.R., Cozzolino, F., Rotilio, G., Garaci, E. and Palamara, A.T. (2003) Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. *FASEB J.* 17: 758-760.
 14. Mikami, T., Satoh, N., Hatayama, I., and Nakane, A. (2004) The inhibitory effect of buthionine sulfoximine on cytopathic effect and apoptosis induced by human echovirus 9. *Arch. Virol.* 149: 1117-1128.
 15. Luo, H., Yanagawa, B., Zhang, J., Luo, Z., Zhang, M., Esfandiarei, M., Carthy, C., Wilson, J.E., Yang, D. and McManus, B.M. (2002) Cocksackievirus B3 replication is reduced by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. *J. Virol.* 76: 3365-3373.
 16. Bhat, N.R. and Zhang, P. (1999) Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. *J. Neurochem.* 72: 112-119.
 17. Barco, A., Feduchi, E. and Carrasco, L. (2000) Poliovirus protease 3Cpro kills cells by apoptosis. *Virology* 266: 352-360.
 18. Agol, V.I., Belov, G.A., Bienz, K., Eggar, D., Kolesnikova, M.S., Raikhlin, N.T., Romanova, L.I., Smirnova, E.A. and Tolskaya, E.A. (1998) Two types of death of poliovirus-infected cells: caspase involvement in the apoptosis but not cytopathic effect. *Virology* 252: 343-353.