The metalloprotease inhibitor EDTA enhances rate of HAV replication in cell culture

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ABSTRACT

Introduction: Hepatitis A Virus is a picornavirus that can be detected in environmental or clinical samples. Its replication in cell culture is slow and so cytopathic effect might take up to two weeks to appear making it not easy to detect presence of virus. the present work aimed to study effect of inhibiting HepG2 cellular proteases on replication rate of the virus. Plaque infectivity count assay was carried out using safe concentrations of the cocktail protease inhibitor MixG and its five components: ABESF, aprotinin, E-64, leupeptin and EDTA. Also quantification of the activities of both intracellular and secreted HepG2 proteases was carried out using specific chromogenic substrate at different ranges of pH values. Results showed that EDTA has intracellular metalloprotease inhibitory effect that enhances rate of HAV replication and thus reduces time needed for the virus to cause cytopathic effect. Such finding will definitely facilitate the detection of the virus both in clinical or environmental samples using cell culture techniques.

Keywords: Hepatitis A virus, HepG2 cells, Protease inhibitors.

INTRODUCTION

Hepatitis A Virus (HAV) is a non-enveloped virus, 27 to 32 nm in diameter that is morphologically indistinguishable from other picornaviruses (Feinstone*et al*., 1973). Its replication in cell culture is slow, persistent and highly asynchronous, and little is known about the rate-limiting steps in the virus life cycle(Yuri *et al*., 2005). Detection of HAV in clinical or environmental samples is not routinely possible because wild-type HAV grows very poorly in cell cultures (Wheeler*et al*., 1986). Except for virus preparations that have been adapted for rapid growth in cell culture, HAV does not produce a detectable cytopathic effect in infected cells (Nasser and Metcalf, 1987).

Various primary and continuous cells of primate origin will support HAV growth, although optimal replication often results from a particular combination of HAV strain, cell type and temperature(Balaian*et al*., 1979). Human hepatoma (HepG2) is one of the commonly used cell lines for HAV isolation and replication. HepG2 cells contain number of cellular proteases including caspases which are cysteine proteases (Toyoda *et al.,* 2002), proteosome which are serine proteases (Cervello*et al.*, 2004) and osteopontin which is secreted matrix metalloprotease (Medico *et al.,* 2001).

Herein we studied the effect of inhibiting cellular proteases using individual and cocktail inhibitors on HAV replication.

Material and methods

Virus: a cell culture adapted strain of Hepatitis A virus-MBB reference strain. Virus was titrated to give final concentration 10^6 PFU/ml.

Virus was kindly provided by Dr Mohamed Ahmed Ali, Prof of virology, water pollution research department, environmental sciences research devision, NRC,Egypt.

Cells: Human hepatoma cells (HepG2 cells) were propagated in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 1% antibiotic-antimycotic mixture. The pH was adjusted at 7.2-7.4 by 7.5% sodium bicarbonate solution. The mixture was sterilized by filtration through nitrocellulose membrane of 0.2 µm pore size.

Protease inhibitors: a) Mix G (serva, Germany): mixture consists of trypsin like serine protease inhibitors (AEBSF-HCL, aprotinin and leupeptin), cysteine protease inhibitor (E-64) and the metallo protease inhibitor (EDTA-Disodium;Serva, Germany).

Cytotoxicity assay

This test was carried out to determine the cell culture safe doses of the protease inhibitors as a mixture or individually **(Aquino *et al*., 1989). HepG2 cells were grown in a 96 well plate an**d were treated with the protease inhibitors at concentrations 3, 5, 10, 15, 20, 25 µg/ 100µl with microscopic observation after 24 hours incubation.

Plaque infectivity count assay:

Plaque infectivity count assay is the most widely accepted method for determining the percentage of affecting virus propagation as a result of being subjected to treatment with a screened material for possible viral count change (Tebas*et al*., 1995). The assay was carried out by two different sequence of steps: a) Briefly HepG2 cells (10^5cell/mL) were cultivated in a 6 well plate and incubated for 1 - 2 days at 37°C. Virus was mixed with the safe concentrations of the compound and incubated for 1 h at 37°C. Growth medium was removed from the multi-well plate and virus-compound mixture was inoculated (100 µl/ well). After 1 h contact time for virus adsorption, the inoculum was aspirated and 3 ml of 1:1 DMEM/agarose was used to overlay the cell sheet. The plates were left to solidify and incubated at 37°C until development of the viral plaques. Formalin was added for two hours to fix the infected cells then plates were stained with crystal violet solution. Control virus and cells wells were treated identically without adding the protease inhibitors. Viral plaques were counted and compared to virus control. B) Cells were prepared the same way as above mentioned, safe concentrations of the protease inhibitors were co-incubated with the cells overnight followed by addition of the virus. After 24 hours plates were subjected to successive freezing and thawing then used to infect new cells, After 1 h contact time for virus adsorption assay was completed as mentioned.

Quantification detection of the proteolytic activity and inhibition (Bahgat*et al*., 2011):

Cells were cultured in 12 well plate at 10^5 cell/ ml and incubated at 37^oc in Co_2 incubator overnight, three control wells were left untreated, EDTA was added at concentration 15 µg/ ml to three groups of wells, first group cells were incubated with EDTA for 24 h, second group for 48 h and third group was inoculated with the virus after 24 h of treatment with EDTA followed by overnight incubation. Media (to measure extracellular proteases) and cells (to measure intracellular proteases) were harvested from all wells and cells were subjected to successive freezing and thawing.

Substrate buffer was prepared (0.17 g Tris, 0.17 g NaCl and 0.17g $CaCl_2$) in 50 ml distilled water then pH was adjusted to either 3, 7 or 9. The specific substrate Bz-

Val-Gly-Arg-*p*-NA (Bachem; Bubendorf, Switzerland).was dissolved at stock concentration of 10 mg/ ml DMSO and diluted with substrate buffer with ratio 1:7 to reach the working concentration. The reaction mixtures (25µl substrate and 50 µl medium or cell lysate) were incubated overnight in a multi-well plate followed by measuring the changes in the optical densities at OD 405.

RESULTS

Cytotoxicity of the used protease inhibitors

The effects of various concentrations of the used protease inhibitors on the viability of the HepG2 cells are summarized in table 1.

Effect of protease inhibitors on HAV count as demonstrated by plaque infectivity count assay

On applying method (a) previously mentioned in the material and methods (M & M) section to test effect of the cocktail protease inhibitor Mix G on HAV replication results (Figure 1A) showed that concentration 3 µg/ ml caused slight inhibition to viral replication but concentrations 5 and 10 µg/ ml enhanced viral replication compared to untreated virus.

Applying method (b) previously mentioned in the M & M section to test effects of various concentrations of the cocktail protease inhibitor Mix G on replication of HAV in HepG2 cells results (Figure 1B) showed slight enhancement of the virus replication at 5 µg/ ml that reached 2.5 fold of the original viral count at 5 µg/ ml.

Using the individual protease inhibitors constituting the cocktail protease inhibitor as well as the Mix G at concentration 5 µg/ ml of results (Figure 2A) showed that both Mix G, E-64 and EDTA could enhance viral count while opposite effects were observed with aprotenin and leupeptin and no change was seen with ABESF. Noteworthy, the increase in virus count due to EDTA reached ~ 3 folds the original count.

On the other hand serial concentrations of the cysteine protease inhibitor E-64 enhanced HAV propagation in HepG2 cells, nevertheless the increase in virus count was inversely proportional to the increase in the inhibitor concentration (Figure 2B). In contrary the enhancement of the HAV propagation was directly proportional to the increase in the EDTA concentration in a linear trend (Figure 2C).

EDTA has an the inhibitory effect of on the intracellular HepG2 proteases that could be quantified using peptide substrate

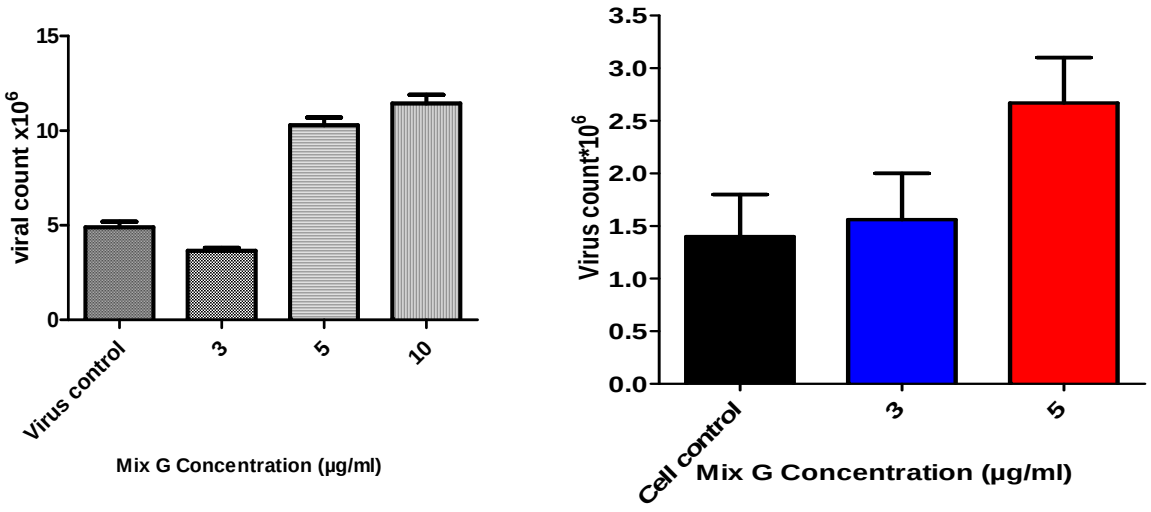
At both neutral and alkaline pH no evident inhibitory effects due to the EDTA were observed either on extracellular or intracellular proteases. While, at acidic pH increasing EDTA concentration clearly inhibited intracellular proteases in presence or absence of the HAV (Figure 3).

Table 1. Cytotoxicity effects of tested protease inhibitors

Compound	Concentration µg/ 100µl					
	3	**5**	**10**	**15**	**20**	**25**
Mix G	+2	+2	+4			
EDTA	Safe					
E-64	Safe					
ABESF	Safe			+2	+4	
Leupeptin	Safe					
Aprotenin	Safe					

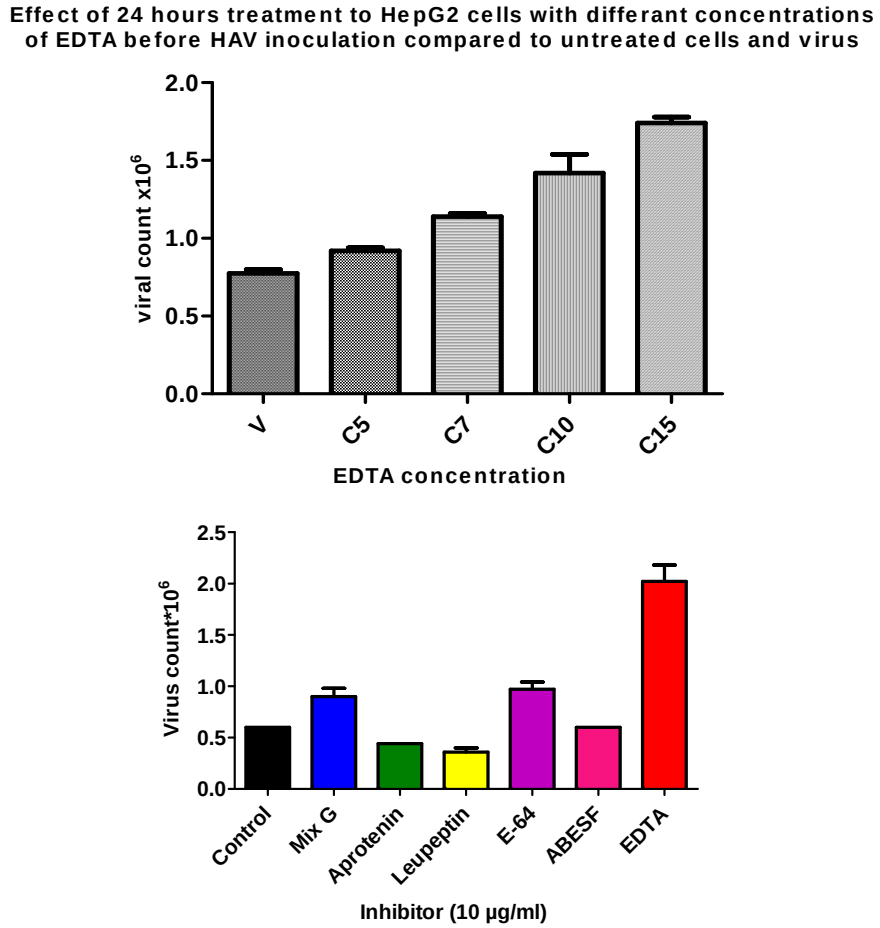
+2: ~50% of cells monolayer is affected.

+4: All cells monolayer was affected.



[A] **[B]**

Figure 1. Enhancement of HAV replication in HepG2 cells by increasing concentration of Mix G in comparison to(A) untreated virus (compound was added with virus at the same time) or (B) untreated cells (compound was added 24 hours before viral inoculation). In virus control experiments (A) Mix G induced HAV replication at concentrations of 5 and 10 µg/ ml, whereas, when treating cells with Mix G 24 hours post infection, experiments (B) the cocktail protease inhibitor caused 2.5 fold increase of the original viral count when applied at concentration of 5 µg/ ml.



[C]

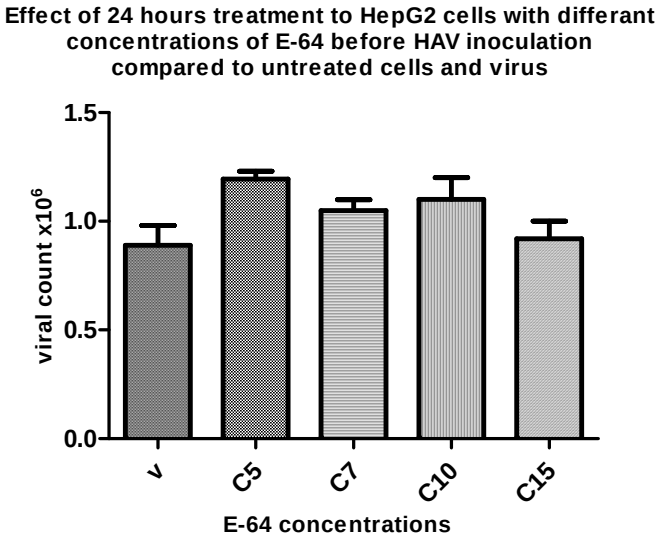


Figure 2: A) Effects of various protease inhibitors on HAV replication in HepG2 cells. Both the cocktail protease inhibitor Mix G, E-64 and EDTA enhanced HAV propagation and the highest count was due to treatment with EDTA. Although both (B) E64 and (C) EDTA enhanced virus propagation, virus count was inversely proportional to E64 concentration (B) but directly proportional to EDTA concentration (C).

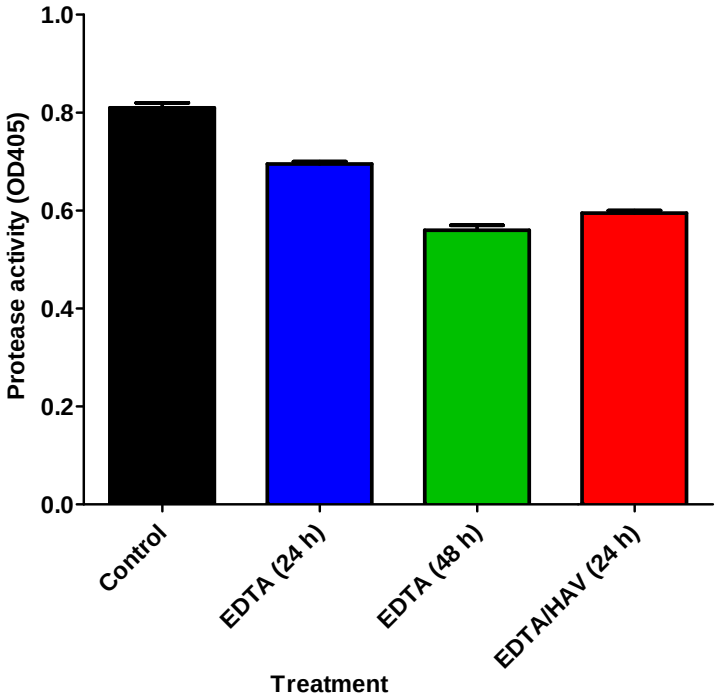


Figure 3. Effect of EDTAon activity of intracellular HepG2 metalloproteases. At acidic pH, EDTA could inhibit the used peptide substrate proteolysis by intracellular HepG2 proteases in absence or presence of HAV.

DISCUSSION

Till present cell culture still represents the only available tool to prove both infectivity and replication of a given virus which makes it superior to either molecular or serological tools which can only report on existence of viral nucleic acids or antigens which do not necessarily mean that the virus is infectious or replicating.

The slow replication rate of HAV in cell culture represents a major challenge facing isolation of the virus from clinical or environmental samples **(3)**. For example, in our hands the wild HAV requires up to two weeks to cause microscopically detectable cytopathic effect (CPE) in HepG2 cells. This is totally non-practical due the following reasons. First, it is extremely non-physiological and stressful to keep mammalian cells for such long duration in culture due to accumulation of catabolic products that can be very toxic for the cells. Second, the cells will be over grown which will make the mission of a slowly replicating virus very hard to get through them.

In fact, poor replication of the HAV is not only hindering the detection of the virus in samples but also blocking vaccine production against pathogenic isolates as they cannot be propagated to the desired titer needed for generation of killed or live attenuated vaccines. Altogether, reflect the urgent need for modifying the conventional cell culture protocols used for propagation of HAV to enable faster virus propagation. One possible approach would be to introducing supplements to the culture medium that might have enhancing effect on HAV propagation.

HepG2 cells express number of proteases including caspases which are cysteine proteases **(6)**, proteosomes which exert both serine and cysteine proteases functions **(7)** and osteopontin which is a secreted matrix metalloprotease **(8)**. In fact our ultimate aim was only to study the effect of the used protease inhibitors on HAV propagation in HepG2 cells as a model RNA virus whose assembly might be controlled by cellular protease. At this stage our expectations did not go beyond this goal. The remarkable increase in resulting HAV plaque count upon treating cells with the cocktail protease inhibitor Mix G at concentrations 5 and 10 µg/ ml of brought direct evidence on the enhancing effect of one or several inhibitors which constitute such a cocktail on virus propagation, nevertheless, it could not nail it down.

Therefore the decision was to study the effect of each of the protease inhibitors constituting the Mix G individually, and results showed that both E-64 (a cysteine protease inhibitor) and EDTA (a chelating agent that inhibits metalloproteinases) remarkably increased HAV propagation while AEBSF (a broad spectrum trypsin like serine protease inhibitor) did not cause any change in viral count, both aprotinin and leupaptin (a general serine protease inhibitor that inhibits both trypsin and chymotrypsin families) caused slight inhibitory effect on viral propagation. The differential effects of individual protease inhibitors of know inhibitory functions against particular classes of proteases enabled nailing down the direct involvement of both cysteine and metalloproteases in HAV propagation and/or assembly.

Although HAV was reported to use the cysteine protease $3C^{pro}$ to catalyze primary and secondary processing steps in its replication cycle **(12)**, in contrary, in our hands the cysteine protease inhibitor E 64 enhanced the HAV propagation at its lowest used concentration. However, further increasing the E 64 concentration was inversely proportional to the HAV count which agreed then with the necessity of cysteine proteases to HAV propagation. A dose dependent inhibitory effect of E 64 on

the mouse hepatitis virus strain A59 was early reported **(13)**.

The direct proportional relation between the concentration of EDTA and the HAV count recorded in the present work clearly pin point the metalloproteinase as the key regulators of the HAV replication and this was further confirmed by the inhibition of the recoded cellular protease activities in presence or absence of HAV by the EDTA at an acidic pH. A possible scenario would be that the inhibited metalloproteinase by the EDTA have a degrading effect on other necessary proteases for HAV assembly, thus, if the metalloproteinase remain active they inactiviate the proteases needed for virus propagation. Another scenario would be that the metalloproteases have possible degrading effects on HAV structural and/or non- structural proteins and thus inhibits formation of HAV virions. Once EDTA is added it inhibits such degrading effects of the matalloprotease and as a result enhances assembly or formation of new virions.

The explanation of recording CPE in HepG2 cells due to HAV after very long duration (~ two weeks) might be referred to accumulation of secondary metabolites that serve as inhibitors for metalloproteases and as result HAV starts to be freely propagating.

In conclusion chelating agents that might serve as metalloprotease inhibitors can be very beneficial in enhancing detection of slowly replicating viruses in cell culture. This is not only limited for diagnosis but might be extended to increase the titer of such viruses to the required levels for generating killed or live attenuated vaccines which can provide protection against infection.

REFERANCES

1. **Aquino, R., DE Simone, C.P., Conti, C., Stein, M.L., 1989.**Plant metabolites, structure and in vitro antiviral activity of Quinovic acid glycosides from Uncariatomentosa and Guettardaplatypoda. journal of natural products. 52:679-685.

2. **Bahgat, M.M., Błazejewska, P., Schughart, K.,2011.** Inhibition of lung serine proteases in mice: a potentially new approach to control influenza infection.Virol J. 20: 8-27.

3. **Balaian, M.S., Andzhaparidze, A.G., Tol'skaia, E.A., Kolesnikova, M.S., 1979.** Possibility of reproducing hepatitis A viral infection in cell systems.VoprVirusol. 6:675-6.

4. **Cervello, M., Giannitrapani, L., La Rosa, M., Notarbartolo, M., Labbozzetta, M., Poma, P., Montalto, G., D'Alessandro N., 2004**. Induction of apoptosis by the proteasome inhibitor MG132 in human HCC cells: Possible correlation with specific caspase-dependent cleavage of beta-catenin and inhibition of beta-catenin-mediated transactivation.Int J Mol Med. 13:741-8.

5. **Feinstone,S..M, Kapikian, A. Z., Purcell, R.H., 1973.** Hepatitis A: Detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science. 182:1026–1028.

6. **Jia, X.Y., Ehrenfeld, E.,Summers, D.F., 1991.** proteolytic activity of hepatitis A virus 3C protein.J Virol.65:2595-600.

7. **Kim, J.C., Spence, R.A., Currier, P.F., Lu, X.,Denison, M.R., 1995.** Coronavirus protein processing and RNA synthesis is inhibited by the

cysteine proteinase inhibitor E64d.Virology.208:1-8.

8. **Medico, E., Gentile, A., Lo Celso, C., Williams, T.A., Gambarotta, G., Trusolino,L.,Comoglio,P.M.,2001**.O steopontin isan autocrine mediator of hepatocyte growth factor-induced invasive growth. Cancer research.61:5861-8.

9. **Nasser, A.M., Metcalf, T.G.,1987**. Production of cytopathology in FRhK-4 cells by BS-C-1-passaged hepatitis A virus. Appl Environ Microbiol. 53:2967-2971.

10. **Tebas, P., Stabell, E.C.,Olivo, P.D.,1995**.Antiviral susceptibility testing with a cell line which expresses beta-galactosidase after infection with herpes simplex virus.Antimicrob Agents Chemother. 39:1287-91.

11. **Toyoda, M., Takagi, H., Horiguchi, N., Kakizaki, S., Sato, K., Takayama, H., Mori, M.,2002**. A ligand for peroxisome proliferator activated receptor gamma inhibits cell growth and induces apoptosis in human liver cancer cells.Gut.50:563-567.

12. **Wheeler, C.M., Fields, H.A., Schable, C.A., Meinke, W.J., Maynard,J.E .,1986.** Adsorption, purification, and growth characteristics of hepatitis A virus strain HAS-15 propagated in fetal rhesus monkey kidney cells. J ClinMicrobiol.23:434- 440.

13. **Yuri, Y.K., Rainer, G.,Verena, G.,2005**. Replication and in vivo repair of the hepatitis A virus genome lacking the poly(A) tail. Journal of General Virology. 86: 1363–1368.

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