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Antiviral function of pyrrolidine dithiocarbamate against influenza virus: the inhibition of viral gene replication and transcription

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Inhibition of influenza virus-induced apoptosis by pyrrolidine dithiocarbamate through antiviral activity: role of reactive oxygen species in apoptosis

The pathogenesis of influenza virus infection involves virus replication in epithelial cells of the respiratory tract and the degeneration of infected cells resulting from subsequent events.[1] Influenza virus induces cellular degeneration following infection of cultured cells *in vitro*, and the cytopathic effect (CPE) occurs principally through apoptotic cell death.[1–10] Influenza virus replication is an essential process for the induction of apoptosis;[3–6] however, the precise mechanism of influenza virus-induced apoptosis is unclear. Recently, several reports have described the effect of antioxidants such as pyrrolidine dithiocarbamate (PDTC), *N*-acetyl-L-cysteine (NAC) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) on influenza virus-induced apoptosis in terms of the involvement of reactive oxygen species (ROS) in a possible mechanism of apoptosis induction (Table 1).[3,7–9,11,12]

Early studies revealed that the effect of antioxidants on CPE has been variable among cell types and strains of influenza virus.[7,8,11] ROS involvement in a mechanism of CPE was obscure. Investigations regarding the effects of antioxidants on CPE and ROS production and virus replication in infected cells have been conducted. Knobil *et al.*[12] reported the inhibitory activity of PDTC on both virus-induced CPE and ROS overproduction; however, antiviral activity of PDTC or NAC was not documented. Recently, Lin *et al.*[9] observed that NAC inhibited apoptosis and ROS overproduction, and virus replication at the early stage of infection, but not overall virus replication; consequently, they claimed that ROS may be involved in the mechanism of apoptosis induction. The role of antiviral activity at an early stage of infection as a possible mechanism of apoptosis inhibition appears to have been overlooked.

Recent studies have elucidated the role of ROS in influenza virus-induced apoptosis employing two different types of cultured cells, two antioxidants and an antiviral agent. Influenza virus infection induced apoptosis and moderate ROS overproduction in cultured chorion cells, whereas decreased ROS production was observed in cultured amnion cells where infection did not induce apoptosis, although virus replicated in both types of cells.[3] These facts provide evidence that influenza virus-induced ROS overproduction is associated with apoptosis; additionally, these findings indicate that the cause is not simply virus replication. PDTC blocked influenza virus-induced apoptosis; however, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid did not.[3] PDTC and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid repressed basal ROS production in mock-infected control cells and virus-induced moderate ROS overproduction, indicating that both PDTC and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid functioned as antioxidants.[3] These observations reveal that the inhibition of ROS overproduction is not accompanied by a blockade of apoptosis; therefore, it appears probable that moderate ROS overproduction is not responsible for the induction of apoptosis. In addition, ribavirin blocked apoptosis and virus replication; in contrast, it did not repress basal ROS production in mock-infected control cells; moreover, ribavirin repressed virus-induced ROS overproduction.[3,4] These facts suggest that ribavirin, although not functioning as an antioxidant, may have repressed virus-induced ROS overproduction by blocking apoptosis as virus replication did not simply cause ROS overproduction. Consequently, moderate ROS overproduction probably occurs as a result of apoptosis induction.

The question relating to how PDTC blocks influenza virus-induced apoptosis has been raised. Metal chelator thujaplicin–copper complex simultaneously inhibited apoptosis and virus replication; moreover, these effects were maintained for up to 2 h post-infection (p.i.).[6] Inhibition of apoptosis by cycloheximide occurred upon addition to the medium within 2 h p.i.; however, inhibition was not efficient at 4 h or later.[10] The latest investigation also revealed that PDTC inhibited apoptosis and virus replication upon introduction to the medium up to 3 h p.i.; in contrast, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid did not inhibit virus replication.[3] These data indicate that virus replication at the early stage of infection plays a critical role in the induction of apoptosis. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid did not inhibit apoptosis or virus replication; however, it did function as an antioxidant. Accordingly, inhibition of influenza virus-induced apoptosis by PDTC is probably attributable to its antiviral activity at the early stage of infection rather than its antioxidant property.

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Table 1. Effects of several chemical agents on influenza virus-induced CPE and their antioxidant properties and antiviral activities

Cell type	Strain of influenza virus	Chemical agent	Inhibition of CPE	Antioxidant property	Antiviral activity	Reference
Chorion[a]	A/Puerto Rico/8/34	PDTC	yes	yes	yes	Uchide *et al.*[3]
		Trolox	no	yes	no	Uchide *et al.*[4]
		Ribavirin	yes	no	yes	
MDCK	A/equine-2/Miami/63	NAC	yes	yes	yes/no[b]	Lin *et al.*[9]
A549	A/Port Chalmers/72	PDTC	yes	yes	no[c]	Knobil *et al.*[12]
		NAC	–	yes	no[c]	
J774.1	X31	PDTC	yes	–	–	Lowy & Dimitrov[11]
		NAC	yes	–	–	
		Trolox	no	–	–	
MDCK	NWS-N8	NAC	yes	–	–	Saito *et al.*[8]
MDCK	A/Turkey/Ontario/7732/66	PDTC	no	–	–	Olsen *et al.*[7]
		NAC	no	–	–	

CPE, cytopathic effect; MDCK, Madin-Darby canine kidney; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetyl-L-cysteine; –, not tested.
[a]Primary cultured chorion cells were prepared from human fetal membrane tissues.
[b]NAC inhibited virus replication at an early stage of infection but not overall virus replication.
[c]Mentioned but data not shown.

Inhibition of influenza virus gene replication and transcription by PDTC: potential antiviral drugs for therapy of influenza

A novel antiviral activity of PDTC against influenza virus is attributable to the blockade of influenza virus-induced apoptosis; however, the antiviral function of PDTC remains unclear. In order to investigate the effect of PDTC on influenza virus gene replication and transcription in infected cells, a novel assay involving semi-quantitative RT-PCR-based methodology and improved by a Southern hybridization technique, has been developed for polarity-specific influenza virus RNAs in cultured cells.[13] The assay revealed that PDTC inhibited influenza virus gene replication and transcription until 6 h p.i.; however, these processes resumed from 12 h p.i., despite the presence of PDTC.[3] A prior exposure of the cells to PDTC intensified its inhibitory effect; however, the duration of this effect was no greater than when PDTC was added at 6 h p.i.[3] Moreover, PDTC eventually delayed and decreased viral protein synthesis.[3] These data indicated that PDTC inhibits influenza virus gene replication and transcription at the early stage of infection. Inhibition of virus replication by PDTC probably results from the inhibition of viral macromolecule synthesis. Moreover, our findings imply that the synthesis of specific viral macromolecules at the early stage of infection may play a critical role in the mechanism of apoptosis induction.

A possible mechanism of the inhibitory effect of PDTC on influenza virus gene replication and transcription is described below. PDTC chelates various divalent metal ions and rapidly recruits zinc and copper ions into cells from the extracellular medium.[14] Zinc or copper ions inhibit influenza virus RNA-dependent RNA polymerase activity; furthermore, the inhibitory effect of metal chelator bathocuproine–copper or bathocuproine–zinc complex is greater than the effect of bathocuproine itself.[15] The metal chelator thujaplicin–copper complex, or $ZnSO_4$, also inhibits influenza virus replication.[6,10] Therefore, it is possible that PDTC inhibits viral gene replication and transcription through the inhibition of influenza virus RNA-dependent RNA polymerase activity by increasing the amount of intracellular copper and zinc ions or intracellular PDTC–copper and PDTC–zinc complexes.

In the event that PDTC acted exclusively as an inhibitor of the replicative enzyme, the viral RNA synthesis terminated by PDTC would not resume in its presence; moreover, prior exposure of the cells to PDTC would not intensify its effect. The present results were contrary to the assumptions; therefore, the mode of action of PDTC appears to be more complex. A maximum level of biological activity of PDTC is known to appear within 15 min and is maintained for more than 3 h.[14] Moreover, it has been confirmed that biological activity of PDTC *in vitro* is not influenced by cytochrome P-450.[16] These facts indicate that PDTC recruits rapidly and is stable in a cell. Accordingly, it seems that kinetics of intracellular recruitment and breakdown of PDTC are not associated with the complexity of its action. On the other hand, cellular proteins such as karyopherin, RNA polymerase regulatory factor, RNA polymerase activating factor and NS1-binding protein are associated with the processes of influenza virus gene replication and transcription; furthermore, PDTC is known to regulate gene expression and/or activity of cellular antioxidant enzymes or transcription factors.[3] Conceivably, PDTC is likely to act not only as an inhibitor of influenza virus RNA-dependent RNA polymerase but also as a modulator of cellular factors associated with viral gene replication and transcription.

In summary, the present findings yield the following conclusions: (i) influenza virus-induced moderate ROS overproduction results from apoptosis and it is not responsible for the induction of apoptosis; (ii) PDTC blocks influenza virus-induced apoptosis via the inhibition of viral gene replication and transcription at an early stage of infection rather than through its antioxidant property; (iii) synthesis of specific viral macromolecules at an early stage of infection may play a critical role in the mechanism of apoptosis induction.

Finally, virus-induced apoptosis can play a beneficial role in cooperation with the host immune system in the host defence mechanism; in contrast, this process can function in a derogatory

capacity, depending on the situation. If virus infection induces massive apoptosis in a broad area of tissue or in essential organs, induction of apoptosis would lead to serious consequences in the infected host. Thus, apoptosis can play a primary role in the pathogenesis of virus. Peptide inhibitors of caspases block the execution of influenza virus-induced apoptosis *in vitro* but not virus replication.[2] Therefore, caspase activation may be involved in the execution of apoptosis by influenza virus infection; however, virus replication may not be directly involved in the process, although it is essential to the induction of apoptosis. It may be said that virus replication is the most important aspect of influenza virus pathogenicity; therefore, genuine anti-apoptosis agents, i.e. caspase inhibitors, may not be suitable for influenza chemotherapy, although these species can block cellular degeneration. Our findings suggest that blockade of influenza virus-induced apoptosis will be achieved via inhibition of viral gene replication and transcription at an early stage of infection as well as via inhibition of virus replication. It is tempting to hypothesize that antiviral drugs such as PDTC may function as future potential anti-influenza drugs.

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