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Intracellular Redox State as Target for Anti-Influenza Therapy: Are Antioxidants Always Effective?

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Abstract: Influenza virus infections represent a big issue for public health since effective treatments are still lacking. In particular, the emergence of strains resistant to drugs limits the effectiveness of anti-influenza agents. For this reason, many efforts have been dedicated to the identification of new therapeutic strategies aimed at targeting the virus-host cell interactions. Oxidative stress is a characteristic of some viral infections including influenza. Because antioxidants defend cells from damage caused by reactive oxygen species induced by different stimuli including pathogens, they represent interesting molecules to fight infectious diseases. However, most of the available studies have found that these would-be panaceas could actually exacerbate the diseases they claim to prevent, and have thus revealed "the dark side" of these molecules. This review article discusses the latest opportunities and drawbacks of the antioxidants used in anti-influenza therapy and new perspectives.

Keywords: Anti-influenza therapy, Antioxidants, Antivirals, Influenza virus, Oxidative stress, Redox state.

1. INTRODUCTION

Influenza virus is a respiratory pathogen contagious to humans, belonging to *Orthomyxoviridae* family that contains three types of viruses (A, B, C). In particular, influenza A virus represents a great serious human pathogen since it causes large recurrent epidemics with high mortality and periodic, unpredictable pandemics.

Influenza virus is an enveloped negative-sense RNA virus and its genome possesses eight segments encoding 10 proteins, and other novel proteins discovered in the last years [1]. The envelope comprises two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the ion channel matrix 2 (M2), that project from the viral surface. Under the envelope, there is the matrix (M1) protein that coats the core of the virus, composed by the ribonucleoprotein (RNP) complex, consisting of the viral genome, the polymerases (PB1, PB2 and PA) and the nucleoprotein (NP). Nonstructural proteins NS1 and NS2 /NEP (Nuclear Export Protein) also constitute the viral particle.

Currently, two options are available to fight influenza: vaccination and antiviral drugs. Vaccination is a key component of defense strategies against influenza, although effective vaccines cannot be produced quickly enough to deal with emerging threats. Anti-influenza drugs include the adamantanes, which target the M2 and inhibit viral uncoating, and NA inhibitors, which block the release of virions from infected cells. Unfortunately, the emergence of strains resistant to antiviral agents highlights the need for drugs that act on new molecular targets, furnishing safe and effective protection against influenza [2]. In this context, targeting of interactions between virus and host cell has been proposed as a novel antiviral strategy that could reduce both viral replication and lung inflammation, as resistance is less likely to occur. Influenza viruses are able to modulate intracellular redox sensitive signaling pathways involved in several cellular functions in order to promote viral replication and pathogenesis [3-8]. Oxidative stress has been described as a characteristic of viral infections that can be caused by several factors, among which the decrease in antioxidant defenses [9, 10] i.e. intracellular glutathione [11-13] and/or the increase in reactive oxygen species (ROS) production [14, 15]. In particular, several papers have reported that ROS and RNS (reactive nitrogen species) contribute to the development of influenza virus-induced pathogenesis in the lung [16-18]. Physiological levels of ROS play a key role in mediating cell signaling, while high levels of ROS can lead to oxidative damage to cellular components and activate several cell death pathways [19]. An "antioxidant defense network" exists inside the host cell to control ROS levels so as to allow useful functions whilst minimizing oxidative damage [reviewed in 20]. For this reason, antioxidants represent interesting molecules that have been proposed for the treat-

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ment of influenza. In 2006, Friel *et al*. [21] proposed the prophylactic use of a carefully designed formulation as nutritional supplement that could counteract the major pathogenic mechanism underlying avian H5N1 influenza in humans.

It would be reasonable to increase antioxidant capacity of the cell using exogenous compounds derived from the diet, thus enhancing cell defenses against the free radical formation. Natural antioxidants present in fruit and vegetables, including vitamins C and E, carotenoids and polyphenols (e.g. flavonoids), are currently considered to be beneficial. In particular, their antioxidant properties are often claimed to be responsible for the protective effects of food components against cardiovascular diseases, some forms of cancer and diseases related to photosensitivity [22]. Other natural compounds have been shown to have antioxidant activity far exceeding the existing antioxidants, i.e. astaxanthin a xanthophyll carotenoid present in different microorganisms and marine animals [23]. Antioxidant enzyme levels, like superoxide dismutase (SOD), catalase, and glutathione peroxidase, were significantly increased in rats after oral dosage of astaxanthin [24, 25]. Furthermore, after dietary supplementation with astaxanthin enhanced antibody production was reported in older animals, suggesting that carotenoid supplementation can be beneficial in restoring humoral immune response [26]. However, many controversies surround the effects of these compounds in experimental models, and their real benefits are still a matter of debate.

This review describes the structure and mechanism of action of the main antioxidants studied as antiviral agents (the relevant *in vitro* and *in vivo* studies are summarized in Table **1**). In particular, this paper discusses the advantages and drawbacks of these compounds used in anti-influenza therapy and focuses on new perspectives.

2. ANTIOXIDANTS AS ANTI-INFLUENZA AGENTS

2.1. Thiol-Based Antioxidants

2.1.1. N-Acetyl-L-Cysteine and Pro-Drugs

N-Acetyl-L-cysteine (NAC) is thiol acting directly as a free radical scavenger and as a precursor of reduced glutathione (GSH). NAC is used as a mucolytic agent and in the treatment of several disorders, including paracetamol intoxication, acute respiratory distress syndrome, bronchitis, AIDS, or psychiatric disorders such as schizophrenia and bipolar disorder [reviewed in 27]. NAC effects are generally attributed to the antioxidant ability of scavenging ROS and increasing intracellular GSH content [28-32], although it has been reported that treatment (oral or intra-peritoneal) with thiols may be associated with increased cysteine levels without concomitant rise in GSH synthesis [33], especially when GSH pools are normal [34]. With regard to viral infections, the protecting activity of NAC was shown in mice infected with influenza A/PR/8 virus, and in *in vitro* models using avian H5N1, a highly pathogenic strain. Indeed, treatment with NAC (1 g/kg per day, orally) significantly decreased the mortality of influenza virus-infected animals [35], and a combination of NAC (1 g/kg) and ribavirin (0.1 g/kg, i.p.) for 4 days reduced the lethal effects induced by influenza virus [36]. Moreover, a combined addition of NAC and oseltamivir, the antiviral usually utilized in the treatment and prevention of influenza, synergistically reduced the lethal effect of influenza virus-infected mice [37]. Recently, NAC was successfully used for a therapy of H1N1 (2009) influenza pneumonia in combination with oseltamivir [38]. These data sustain the notion that combination of antioxidant therapy with available drugs can improve the treatment of influenza.

In *in vitro* studies, the continuous treatment with NAC, starting by 24 h pre-incubation, reduced cytopathic effects and apoptosis induced by H5N1 strain, as well as viral titer 24 h post-infection [39]. NAC also decreased production of pro-inflammatory cytokines [CXCL8, CXCL10, CCL5 and interleukin (IL)-6] in alveolar type II epithelial (A549) cells infected with H5N1 influenza virus. Specifically, the antiviral and anti-inflammatory mechanisms of NAC included inhibition of redox-sensitive pathways activation, among which transcription nuclear factor (NF)-κB and p38 mitogen activated protein kinase (MAPK) [39]. Proteomic studies performed by Wu and collaborators [40] demonstrated that NAC was able to protect PR8 infected lung epithelial cells from apoptosis induced by the virus. In addition, Mata *et al.* [41] demonstrated that administration of NAC inhibited the production of mucin (MUC5AC) and the expression of pro-inflammatory cytokine in A549 cells infected with different viruses, including influenza A, B or respiratory syncytial virus (RSV). In these experiments, NAC decreased the hydrogen peroxide (H_2O_2) production and restored intracellular total thiol levels depleted by viruses. These effects were associated with significant but weak decrease in viral titer by 10.31%, 12.99% and 30.12% for RSV, influenza A and B viruses, respectively.

Although these positive results, Garigliany and Desmetch [42] reported that NAC lacks universal anti-influenza activity. Indeed, these authors showed that NAC was not able to change the course of a fatal influenza pneumonia caused by infection with a murinized swine H1N1 influenza virus. Moreover, NAC treatment inhibited the swine viral replication *in vitro* but lesser than reported for other strains. Therefore, the authors suggested that anti-influenza activity of NAC seemed to be strain-dependent, and for this reason NAC could not be universally used for influenza pneumonia. Our preliminary *in vitro* results in lung epithelial cells indicate that despite NAC was able to restore the intracellular redox state perturbed by viral infection, its antiviral activity was very limited when the treatment was done after viral challenge. Surprisingly, although the compound was able to inhibit virus-induced p38 MAPK phosphorylation, it exacerbated the pro-inflammatory cytokines production (Sgarbanti, personal communication). In conclusion, the beneficial effects of NAC on cytokine production and on pathological conditions are still controversial [43-47]. Recently, it has been demonstrated that supplementing the diet with NAC and vitamin E markedly increased the progression of tumor in mice and reduced survival of B-RAF– and K-RAS–induced lung cancer by disrupting the ROS-p53 axis. This may be relevant to patients with chronic obstructive pulmonary disease, who are often smokers with increased risk of developing lung cancer, and they ingest high amounts of NAC to relieve mucus production [48]. Despite the large use of NAC, its administration has some drawbacks such as low systemic bioavailability; for this reason some pro-drugs of

Table 1. Relevant *in vitro* and *in vivo* findings on antioxidants proposed as anti-influenza agents.

Thiol Compounds and Pro-Drugs	Effect on Different Models of Influenza Virus Infection	
	In vitro	***In vivo***
N-acetyl-L-cysteine (NAC)	Twenty-four h pre-incubation (5 to 15 mM): reduction of H5N1 virus-induced cytopathogenic effects, apoptosis and viral titer; reduction of pro-inflammatory molecules [39, 40]. Pre- and post-treatment: decrease of pro-inflammatory cytokine production; weak decrease of viral titer; correlation with antioxidant activity [41]. Post-treatment (15 mM): partial protection against H1N1 (2009) virus [42].	Oral or IP treatment (1g/Kg per day): reduction of influenza virus-induced lethal effects (alone or in combination with anti-viral) [35-38]. Oral administration (100 mg/Kg): no protection against H1N1 (2009) [42].
Glutathione (GSH)	Post-treatment (10 mM): strong reduction of viral titer and protein expression; increase of intracellular GSH levels in infected cells [12, 13].	Oral administration (50 mM): decrease of viral titer in both lung and trachea homogenates [13].
GSH-C4	Post-treatment (7.5-10 mM): strong reduction of viral titer; impairment of influenza HA maturation; increase of intracellular GSH levels [11].	IP treatment (370 mg/Kg): reduction of mortality, viral titer in lungs and virus-induced inflammation [11].
PDTC	Post-treatment: inhibition of HA viral RNA, virus-induced apoptosis and ROS overproduction [72].	IP pre-treatment (75, 150, 200 mg/Kg): strong increase of survival; decrease of viral titer in lungs [71].
Polyphenols		
Resveratrol (RV) and analogue	Post-treatment (20 μg/ml): strong decrease of viral titer; no correlation with antioxidant activity [90]. Post treatment with analogue (10 μg/ml): impairment of vRNP traffic; partial restore of virus-induced GSH depletion [95].	IP treatment (1 mg/kg/day): significant improve of survival; decrease of pulmonary viral yields [90].
Curcumin and analogue	Pre-treatment (30 μM): strong reduction of virus yields [96]. Post-treatment (20 μg/ml) with analogue: block of NP in the nucleus; inhibition of late protein synthesis; impairment of HA maturation; restore of reducing condition in the cells [95].	N.A.
Hydroxytyrosol	Post-treatment with catechol derivatives of hydroxytyrosol (IC50=30 μM): reduction of viral titer [97].	N.A.
Tocopherols		
Vitamin E	N.A.	IP pre-treatment (60, 120, 240 mg/Kg): protection from increased virus induced-lipid peroxidation [113, 114]. Aged-mice fed with 500 ppm (supplemented diet): increase of antiviral activity; enhancement of Th1 response [115, 116]
Trolox	Post-treatment: decrease of ROS overproduction; no inhibition of viral titer and virus-induced apoptosis [72].	N.A.
Vitamin C	Weak inhibition of viral replication; Treatment with dehydroascorbic acid (40 mM): strong antiviral activity due to toxic effects [108].	N.A.
Enzymes		
NADPH oxidase (NOX)	Treatment with NOX4 inhibitor or NOX4 silencing: reduction of viral titer; inhibition of ROS production [14].	NOX2-deficient mice: significant reduction in lung injury; improvement in lung function; lower airway inflammation and alveolar epithelial apoptosis [134, 137].
Superoxide dismutase (SOD)	N.A.	Modified Cu/Zn SOD: reduction of lethality of infection [141]. Naturally glycosylated Cu/Zn SOD in combination with rimantadine or polyphenol-rich extract: decrease of mortality rates and lung viral titer [142, 143]. Aerosol of MnSOD in combination with ribavirin: inhibition of infection [144].

IP: intraperitoneal administration
N.A.: Not available

NAC were synthesized. An example is represented by I-152, a pro-drug of NAC and cysteamine. Upon esterase activation, it can release both products and NAC can be used to increase intracellular GSH levels [49]. Antiviral activity of I-152 was demonstrated in *in vitro* and *in vivo* models [49, 50].

2.1.2. Glutathione (GSH) and Analogues

Influenza virus induces a depletion of GSH [11-13], the most abundant intracellular antioxidant, which plays a key role in maintaining the redox state [51] and in scavenging ROS [52]. Many authors demonstrated that the replenishment of intracellular GSH obtained with the administration of GSH, GSH derivative or GSH precursor inhibited the viral replication of several viruses *in vitro* and *in vivo* [3, 53]. Cai *et al.* [13] demonstrated that GSH administration inhibited viral matrix protein expression, caspase activation and Fas upregulation in influenza virus-infected MDCK cells. The GSH addition in the drinking water decreased viral titer in lung and trachea homogenates, 4 days after infection with a mouse-adapted influenza A/X-31 strain. Since the GSH is a molecule not readily transported into most cells or tissues, the n-butanoyl GSH derivative (GSH-C4) was tested for its antiviral activity against different viruses [54, 55].

Sgarbanti *et al.* [11] reported that GSH-C4 is effective against influenza virus. In particular, it interfered with the viral glycoprotein HA maturation, a process mediated by redox-sensitive activity of protein disulfide isomerase (PDI), a cell oxidoreductase in the endoplasmic reticulum (ER) (Fig. **1**). In fact, the oxidative environment established by GSH depletion during viral infection, is needed to increase the expression and oxidation of PDI, thus accelerating disulfide bonding and enhancing HA maturation. Accordingly, treatment with oxidant compounds, including buthionine sulfoximine BSO (an inhibitor of GSH synthesis), cadmium, morphine or cocaine, significantly decreased intracellular GSH content and increased HA expression and viral yields measured in supernatants of infected cells [11, 12, 56-58]. The protective effect of GSH-C4 has been demonstrated in a mouse model of influenza virus infection, where it reduced lung damage and mortality [11]. Since the thiol moiety of GSH is important not only in the antioxidant defense but also in the selective inhibition of viral protein disulfide bond formation (acting as "chemical blocker" of folding process), we suggest that the research should address more about the metabolic processes that contribute to the maintenance of this tripeptide within cells. It is useful to taken into account that treatment with pro-GSH molecules, including GSH-C4, increases *in vitro* and *in vivo* the intra-macrophage thiol content and it regulates the shift of immune response towards T helper (Th) 1-type response, which plays a key role in antiviral immunity [59]. In fact, in Ovalbumin-immunized mice the increased intra-macrophage thiol pool after pro-GSH molecules addition, modulated the Th1/Th2 balance in favor of a Th1 response [60]. These results together with those obtained in animals immunized with HIV Tat (Trans-

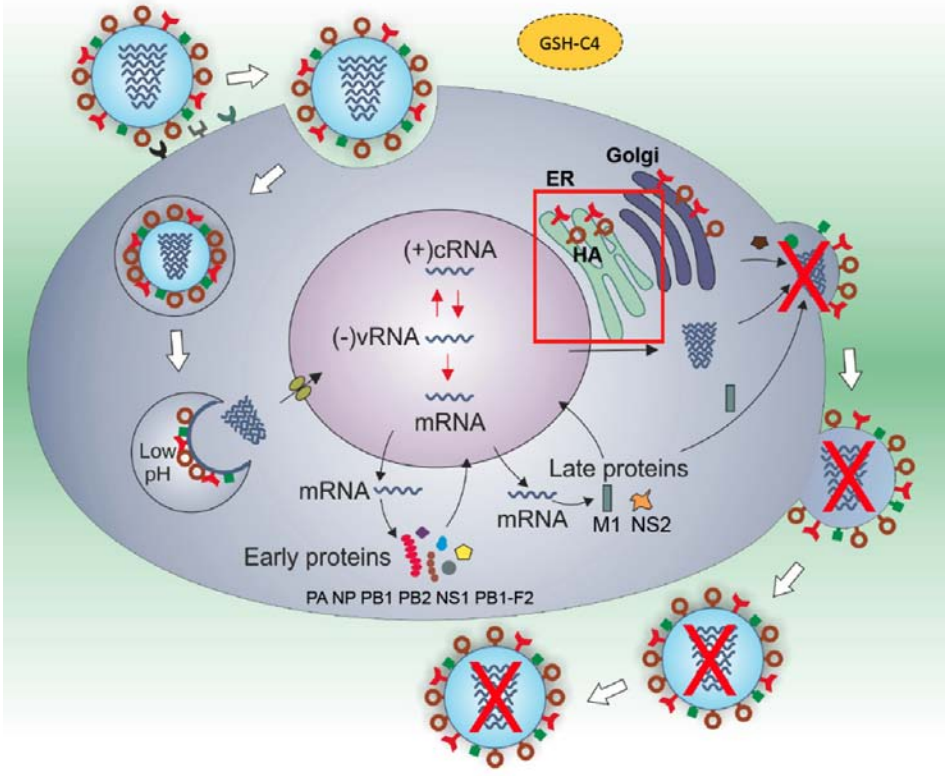

Fig. (1). Proposed molecular mechanism of GSH-C4 in inhibiting influenza virus replication. GSH-C4 arrested the folding of viral hemagglutinin (HA): this disulfide-rich glycoprotein remained in the endoplasmic reticulum (ER) as a reduced monomer, instead of undergoing oligomerization. As a consequence, its insertion into the cell plasma-membrane was strongly reduced and the virus release was blocked. The mechanism underlying GSH-C4 antiviral effect is related to the host-cell oxidoreductase, protein disulfide isomerase (PDI). This protein works in ER by helping the formation of disulphide bond during glycoprotein maturation [11].

activator of transcription) [61] suggest that pro-GSH molecules could be useful for modulating the immune response towards different antigens and could be further exploited in new vaccination protocols for inducing specific Th1 immune responses against intracellular pathogens or as immunomodulators in some diseases where Th1 response patterns are compromised in favor of Th2. Other GSH analogues were used to enhance macrophage Th1 cytokine production. Given the important role of GSH content in the antigen presenting cells in inducing differentiation of Th population into either Th1 or Th2 phenotype, some authors used GSH mono ethyl ester to enhance the Th1 cytokines production, such as IL-12 and IL-27, in macrophages and dendritic cells [62, 63]. To the same aim, other authors used NAC [64]. As already observed elsewhere regarding the pro-oxidant or antioxidant effect of antioxidants during viral infection, the same molecules may specifically induce different sets of genes controlling Th1 cytokine expression depending on the type and redox status of the cell; furthermore, the same molecules can exert different effects according to the concentration used [64].

2.1.3. Pyrrolidine Dithiocarbamate

Pyrrolidine dithiocarbamate (PDTC) is a thiol-containing molecule that can function either as pro- or anti-oxidant compound depending on the experimental conditions. The antioxidant properties of PDTC are attributed to its ability to scavenge radicals, to chelate ions and to alter ROS metabolism. In addition, PDTC regulates antioxidant enzyme gene expression, including manganese superoxide dismutase (MnSOD) [65], heme oxygenase-1 [66] and γ-glutamylcysteine synthetase [67] but, conversely, it may act as a pro-oxidant and modulator of free thiol groups [68, 69]. PDTC has been shown to be an inhibitor of NF-κB, due to its ability to traverse the cell surface and its prolonged stability at physiological pH [70]. For this reason, PDTC has been proposed for the treatment of acute and chronic inflammatory conditions in which NF-κB activation plays a major role [71].

It has been reported that PDTC was able to suppress ROS accumulation induced by influenza virus infection in chorion cells [72]. PDTC inhibited both apoptosis induction and viral replication in the infected cells, whereas no such inhibitory effect was observed after Trolox treatment. For this reason, the authors suggested that ROS production could not be responsible for influenza virus induced-programmed cell death. On the contrary, they suggested that this inhibition could be attributable to the antiviral activity of the compound rather than its antioxidant properties. Other authors demonstrated that PDTC inhibited the cytopathic effect of influenza virus infection on other types of cells, such as A549 and murine macrophage J774.1 cells [73-75]. Recently, Wiesener *et al.* [76] described the protective effect of PDTC in mice infected with a mouse-adapted strain of A/PR/8/34 (H1N1) simultaneously treated with PDTC [75, 150, 200 mg/kg body weight (b.w.), intraperitoneally]. The treatment increased survival up to 80% and reduced weight loss and virus titer in lung tissue in a dose-dependent manner. The efficacy was less pronounced, if the treatment started later during influenza A virus infection. Moreover, simultaneous treatment with PDTC limited infiltration of immune cells as well as local interferon (IFN)-γ expression in lung tissue.

Although PDTC is an anti-oxidant, some authors suggest that this characteristic can not be responsible for its ability of inhibiting NF-κB in tubular epithelial cells [77]. On the contrary, the pro-oxidant and metal-chelating properties of PDTC could paradoxically be involved in its ability of inhibiting the transcription factor [78]. Accordingly, PDTC seems to act catalytically at micromolar concentrations and cause the oxidation of several hundred molar equivalents of GSH [68, 78].

2.2. Polyphenols

Polyphenols are largely diffused in the plant kingdom, especially in fruits (like berries, pomegranate, and apple), nuts and vegetables as well as beverages, including green tea and red wine [79, 80]. They have a plethora of therapeutic health effects for many diseases including cancer, neurodegenerative diseases, diabetes, cardiovascular diseases and infectious diseases [81-83]. Particularly, polyphenols and their semi-synthetic derivatives have been shown to exert anti-inflammatory as well as anti-influenza activity [81, 84-86]. The anti-influenza activity of polyphenols has been related to their antioxidant property. Indeed, a polyphenolic extract from the medicinal plant "*Geranium sanguineum* L." exerts anti-influenza activity and antioxidant and radical scavenging properties [87]. Later the same authors demonstrated that these extracts could act not only as antioxidants, but also as pro-oxidants, thus indicating a dual characteristic of some polyphenols. In fact, the biological properties of polyphenols may be both antioxidant and/or pro-oxidant based upon the structure of the particular polyphenol and the cellular redox context that may include increased levels of oxidant scavenging proteins or decreased levels of oxidized proteins and lipids [83]. Among polyphenols, resveratrol (trans-3,4,4'-trihydroxystilbene, RV) is a stilbene-like phytoalexin present in more than 72 plant species, among which grape skin and other fruits, with high antioxidant activity [88]. It plays a relevant role in several diseases including viral infections [89]. We previously demonstrated that RV inhibited influenza A virus replication by acting on cellular pathways involved in the regulation of specific steps of virus life-cycle, the nuclear-cytoplasmic traffic of viral RNP complex and the late viral protein synthesis [90]. We also confirmed RV's antiviral effects *in vivo* model. In fact, treatment of influenza virus-infected mice markedly increased survival and decreased lung virus yields. We suggested that different mechanisms could underlie the *in vivo* efficacy of RV, including inhibition of both virus titer and NF-κB-induced inflammation. In fact, it is known that RV inhibits several cell pathways that are involved in the inflammatory airway damage, characteristic of influenza [91]. Furthermore, it has been reported that (+)-vitisin A, a tetramer of RV, potently inhibited RANTES production by interfering with Akt- and STAT1-related signal pathways during influenza virus infection [92]. Finally, RV might be involved in the regulation of innate response. In fact, Xie *et al.* [93] reported that RV decreased IL-6 production and partially inhibited RSV replication in cell cultures, and this might be

related to an inhibitory effect on TIR-domain-containing adapter-inducing interferon-β (TRIF) complex. Interestingly, in our model no correlation between RV's antioxidant and antiviral activity was observed. Indeed, during influenza virus infection, RV treatment was not able to restore GSH depletion and, in mock-infected cells, it decreased the intracellular GSH content (compared with untreated cells) [90]. These results can be explained because RV may produce *in vivo* anti-oxidant or pro-oxidant effects depending on its oxidative status, that in turn mirrors the redox potential of the microenvironment. Thus, while RV can quench reactive free radicals by donating hydrogen atoms, this process generates phenoxyl radicals that can oxidize GSH to GS•. Furthermore, oxidation of the RV-phenoxyl radical produces a quinone form, that can alkylate GSH, further decreasing intracellular levels of free GSH [94].

Differences in anti-influenza activity between neo synthesized analogues of RV and curcumin have been reported by our group [95]. Curcumin [diferuloyl methane, 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione] is a natural polyphenol present in the rhizome of *Curcuma longa* L. known to be effective against influenza virus [96]. In our study, we demonstrated that both analogues inhibited viral replication by impairing vRNP traffic, nevertheless while RV analogue partially interfered with redox state of host cell, curcumin analogue prevented virus-induced GSH depletion. As a consequence, redox-sensitive pathways involved in viral HA maturation and localization on cell surface could be impaired [95]. Interestingly, curcumin analogue brought two catechol groups, usually known to contribute to the antioxidant activity of compounds. Bozzini *et al.* [97] demonstrated that catechol derivatives with lipophilic properties exerted different anti-influenza activity depending on their length of carbon alkyl side-chain. The best compounds were catechol derivatives of hydroxytirosol, with significant antioxidant activity and relatively long carbon alkyl side-chain, suggesting that the anti-influenza activity could be due to two main factors, the antioxidant property of catechols and the presence of a lipophilic side chain (more than two carbon atoms).

Accordingly, our previous data showed that GSH-C4, a lipophilic GSH derivative, was able to enter into the cells more easily than GSH, thus inhibiting more effectively the replication of DNA and RNA viruses [11, 54].

2.3. Ascorbic Acid

Ascorbic acid, or vitamin C, has been widely suggested as antiviral agent, especially against influenza virus. High doses of vitamin C for the prevention and treatment of colds have been proposed in 1970 when the Nobel Linus Pauling published the book "Vitamin C And The Common Cold".

In 1999, controlled clinical trial demonstrated that vitamin C in megadoses (1g, 3 doses daily) given before or after the appearance of cold and flu symptoms mitigated and prevented the symptoms in the population compared with the control group [98]. However, the role of vitamin C in the prevention and treatment of the common cold has been a subject of debate for at least 70 years.

Cochrane review [99] on this topic disclosed the failure of vitamin C supplementation in reducing the incidence of colds in the general population thus indicating that routine vitamin C addition is not justified, even if vitamin C can be useful for people exposed to brief periods of severe physical exercise. A discordance of the use of this vitamin comes from some trials that show that regular supplementation of vitamin C reduced the duration of colds, while other trials did not show the same results.

As reviewed in Yuan [100], high amounts of vitamin C would be helpful for patients during a severe avian influenza. Thorson *et al.* [101] demonstrated that in different public places, such as Vietnam, about 50% of the humans infected with avian flu (H5N1) did not die. Ely [102] proposed that their survival could be occurred because infection could be moderate enough to be counteracted by vitamin C, accidentally acquired from diet. It is known that 5 mM of vitamin C (about 4.4 g for about 5 L of human blood) is the effective dose to inhibit viral replication and apparent symptom alleviation usually requires over. However, it should be considered that over 1 g of vitamin C by oral administration can cause diarrhea, nausea, vomiting, stomach cramps and other side effects [reviewed in 100]. The protective effect of ascorbic acid is probably due to i) its potent scavenging and antioxidant property and ii) its accumulation in millimoles per liter in neutrophils, lymphocytes and monocytes [103, 104]. It has also been demonstrated that Vitamin C is an essential factor on the antiviral immune response at the early time of infection, through increased anti-viral cytokine IFN-α/β production. This effect has been suggested in vitamin C-insufficient Gulo (-/-) mice infected with influenza virus (H3N2/Hongkong). These animals died within 1 week after intranasal infection with influenza virus, viral yields in the lung were definitely increased and production of IFN-α/β was decreased. Moreover, the inflammatory cell infiltration into the lung and pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and IL-1α/β, production were increased [105].

Vitamin C is considered a powerful antioxidant and intervenes in several physiological processes, but it can also act as a pro-oxidant when it reacts with iron or copper, which in turn reduces hydrogen peroxide to hydroxyl radicals [106, 107]. Furuya *et al.* [108] demonstrated that ascorbic acid weakly inhibited viral replication of several viruses, including influenza. A much stronger antiviral activity was observed by dehydroascorbic acid, an oxidized form of ascorbic acid, thus indicating that the antiviral activity was probably due to cytotoxic effects than that to its antioxidant property.

2.4 Vitamin E and Analogues

Vitamin E (α-tocopherol) is the common term given to a group of fat-soluble compounds, which possess different antioxidant activities essential for human health [109]. The human diet contains eight different vitamin E-related molecules including α, β, γ, δ-tocopherols and tocotrienols which are synthesized by plants. Although these molecules are peroxyl radical scavengers, the human body prefers α-tocopherol [109]. As concern the tocopherols, the α- and γ-tocopherols are found in the serum and red blood cells, with

the α-tocopherol present in the highest concentration [110]. The α-tocopherol form accumulates particularly at sites where free radical production is greatest, such as in the membrane of mithocondria and ER in the heart and lungs [111]. It acts as the first line of defense against lipid peroxidation, protecting the cell membranes from radical attack. In particular, α-tocopherol mainly inhibits the production of new free radicals. Since oxidation has been linked to numerous diseases including viral infections, vitamin E might represent a good tool for the treatment of ROS-associated diseases. It is known that influenza virus infection in mice causes a decrease in levels of the antioxidant nutrients [112-114]. In particular, it has been reported that influenza virus causes a marked increase of lipid peroxidation products in the liver, blood and lung of infected mice accompanied by a decrease of vitamin E content [113, 114]. Supplementation with exogenous vitamin E (60, 120, 240 mg/Kg b.w.) before virus infection protects mice against lipid peroxidation. Indeed, in these conditions, a decrease of lipid peroxidation products and an increase in vitamin E content were established. The effect of vitamin E was dose-dependent in blood and liver while in lung tissues it was dose-independent, probably due to their different fatty acid and phospholipid composition [113, 114]. Aged-mice supplemented with 500 parts per million (ppm) of vitamin E had significantly reduced lung viral titers, with respect to old mice fed with a diet containing adequate levels of vitamin E (30 ppm) [115]. Interestingly, vitamin E was more effective in reducing viral titer in old mice than in young mice, probably due to the fact that aging is associated with increased oxidative stress. Therefore, the authors suggested that influenza virus-induced oxidative stress in aged-mice might require higher levels of antioxidant nutrients to control viral replication to the same level as in young animals. The antiviral effect of vitamin E was mediated by an improvement of the Th1 response, which was impaired in influenza virus-infected old mice [116]. The effect of natural tocopherols on the regulation of redox balance in the cells depends on the presence of the corresponding tocopherylquinones. Indeed, quinones can act as potent electrophiles altering the internal redox potential of the cells. Saladino *et al.* [117] evaluated the antiviral effect of natural tocopherols and the corresponding tocopherylquinones and cathecols in *in vitro* model of influenza virus infection. Interestingly, only the reduced form of tocopherols was able to inhibit viral replication indicating a key role of the oxidation state of the molecule on the antiviral activity. Importantly, in some trials using vitamin E supplementation there was an increased mortality [118]. As reviewed in Villanueva *et al.* [119], antioxidants become "unstable" and "reactive" when they lose or receive electrons in the presence of reactive species. In particular, α-tocopherol produces α-tocopheroxyl radical when it reacts with reactive species like peroxynitrite [120] or superoxide [121], and it is converted to α-tocopherol by other antioxidants among which vitamin C and GSH [122-124]. When ascorbic acid recycles vitamin E, it is transformed to the ascorbyl radical, which is less reactive than α-tocopherol [122]. Therefore, the authors suggest that vitamin E should be provided with other antioxidants. Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) is a cell-permeable, water-soluble vitamin E analogue with potent antioxidant properties. The carboxyl group present within the structure gives water solubility, which renders the use of Trolox more advantageous respect to other active antioxidants (e.g. vitamin E) that are only lipid-soluble [125]. Trolox has been used for antioxidant therapy in different models in which ROS are formed, including myocardial injury and diabetic retinopathy.

In primary cultured chorion cells isolated from human fetal membranes infected with influenza A PR8 virus, Trolox (500 μM) was able to inhibit the virus-induced ROS production but it did not inhibit DNA fragmentation and viral replication [72]. Therefore, Trolox seems to act as antioxidant but not as antiviral. In MDCK cells infected with influenza virus A H3N2, Trolox added in combination with rimantadine did not show any pronounced protective effect [126]. Our preliminary experiments on human lung epithelial cells infected with PR8 virus demonstrated that Trolox treatment after viral adsorption did not block the release of viral particles from infected cells compared to untreated infected ones, and exacerbated the virus-induced pro-inflammatory cytokines production (Sgarbanti, personal communication). The mechanism of this increased cytokine production could be explained by the fact that Trolox may be oxidized by a variety of free radicals to the phenoxyl radical [127], then the cells' environment may remain oxidized thus favoring the progression of influenza virus infection.

2.5. New Strategies for Inhibiting ROS Production

2.5.1. NOX Inhibitors

As previously described, physiological levels of ROS interact with redox state and play a role in activating signaling cascades involved in several cell functions including growth and differentiation [for review see 128]. On the contrary, excessive cellular generation of ROS is pathological and potentially destructive and can result in oxidative damage to cellular components [19]. Several enzymes in the cells are able to produce ROS among which xanthine oxidase [129], cytochrome P450 oxidase [130], uncoupled nitric oxide synthase [131], NADPH (nicotinamide adenine dinucleotide phosphate) oxidases [132], and the mitochondrial electron transport chain [133]. However, only NADPH oxidases produce ROS as their primary and unique function. These enzymes are multi-protein complexes consisting of a catalytic, transmembrane-spanning subunit (NOX), as well as various structural and regulatory proteins localized on the membrane and in the cytosol. NOX family comprises seven members, NOX1-5, and two dual oxidases (Duox), Duox1 and Duox2 functionally expressed in different tissues and organs [132]. NOX enzymes differ in enzymatic composition, modes of activation, and the products of their enzymatic reaction. NOX isoforms regulate different physiological process but they are also implicated in several diseases including viral infections [132, 134, 135]. Regarding influenza virus, it is known that Nox2-derived superoxide production is responsible for the pathogenesis of infection [136]. In particular, Snelgrove *et al.* [134] showed a significant reduction in lung injury and improvement in lung function after influenza virus infection in NOX2-deficient mice. At the same time, Vlahos *et al.* [137] demonstrated that influenza A virus infection increases the NOX2-derived superoxide production that is responsible for increasing of peroxynitrite formation

in the lung, thus contributing to the lung injury. Moreover, NOX2-deficient mice showed lower airway inflammation and alveolar epithelial apoptosis after infection with influenza viruses at low and high pathogenicity [137]. Besides the implication of NOX2 in inducing lung inflammation in infected mice, we have reported a role for NOX enzymes also in viral replication by controlling specific steps of influenza virus life-cycle [14]. In particular, we demonstrated that influenza A virus infection transiently increased intracellular ROS in lung epithelial cells. This process led to the activation of the p38 and ERK1-2 MAPK pathways that, in turn, supported the nucleo-cytoplasmic traffic of vRNP, a key event for viral assembly and release. In human pulmonary cell lines and in murine primary airway epithelial cells, NOX4 was the prime actor in the virus-induced oxidative stress and, as a consequence, in favouring viral replication. NOX4 expression was up-regulated during infection, while chemical inhibition or knockdown of NOX4 significantly impaired the release of viral particles from infected cells. Because of the lack of specificity of antioxidants toward a certain ROS at a specific site and the clinical failure of antioxidant treatments, NOX enzymes may represent a good strategy for the treatment of diseases associated with oxidative stress including viral infections. Several NOX inhibitors are currently available, but they lack clear NOX isoform selectivity [138]. Therefore, future NOX inhibitors characterized by selectivity for specific isoforms would be of great interest.

2.5.2. Superoxide Dismutase

Superoxide dismutases (SODs) are metalloproteins that dismutate the superoxide radical (O_2^-) into H_2O_2 and molecular oxygen (O_2).

Mammalian cells are characterized by a SOD enzyme in the mitochondria that contains active site manganese (MnSOD) and a SOD with active site copper and zinc (CuZnSOD) largely present in the cytosol [139]. Recently, high levels of oxygen free radicals (OFRs) and decreased SOD activity were found in lungs of mice infected with avian H5N1 strain. Thus, the authors suggested a role of OFRs in acute lung injury caused by this virus [140]. Akaike *et al.* [141] demonstrated the pathogenic role of O_2^- induced by a cascade of adenosine catabolism in influenza virus-infected mice. Specifically, it was generated in Bronchoalveolar lavage fluid of influenza virus-infected mice because of an elevated xanthine oxidase (XO) and its substrate, as a result of increased levels of adenosine catabolites such as hypoxanthine and xanthine. The elimination of oxygen radicals through the treatment of infected mice with allopurinol (a XO inhibitor) and with chemically modified SOD (CuZn SOD conjugated with a pyran copolymer) had therapeutic effects by reducing the lethality of infection. Free CuZn SOD did not exhibit protective activity, because of its short pharmacokinetic clearance time. The effect of a naturally glycosylated CuZn SOD, produced by the fungus *Humicula lutea* (HL-SOD) strain 103, was evaluated in combination with rimantadine hydrochloride in protecting mice by influenza virus [142]. While the single treatment did not significantly protect mice against the infection, HL-SOD and rimantadine combination decreased lung viral titers, lung weights and mortality rates, and prolonged survival times. Interestingly, similar results were obtained with the combined application of HL-SOD with a polyphenol-rich extract, isolated from *Geranium sanguineum* L. [143]. An inhibitory effect on influenza virus infection has been demonstrated by the treatment of infected mice with MnSOD an enzyme with a longer plasma clearance time (half-life [t1/2], about 6 h in mice), in combination with ribavirin each administered with small-particle aerosol [144]. However, the authors reported that MnSOD effects were virus dose-dependent. Indeed, weak inhibition of mortality was observed in mice infected with high doses, while strong inhibition occurred in animals infected with low doses. Finally, Suliman *et al.* [145] reported that the enhancement of extracellular SOD in the conducting and distal airways in lung of transgenic mice minimized lung injury caused by influenza virus by reducing inflammation and impairing oxidative stress.

2.5.3 Mitochondrially Targeted Compounds

Among the potential sources of ROS discussed above, mitochondrial ROS (mROS) have attracted attention since it has been recently discovered that they contribute to inflammatory cytokine production and innate response [146] by activation of specific intracellular pathways [reviewed in 147]. However, at the present antioxidants are not selective for mitochondria and this fact may hamper their effectiveness [148]. The "ideal" antioxidant should be specifically targeted to mitochondria where ROS are produced and it should effectively remove not all the ROS but just their excess. It is also important for an antioxidant not to be toxic and not to be recognized and eliminated by cell enzymes, as described in the SkQ project - organized in participation with several research groups and aimed at the synthesis of a new type of compounds (SkQs) including plastoquinone (an antioxidant moiety), a penetrating cation, and a decane or pentane linker [148]. Between potential mitochondrial protective drugs, it should be taken into account mitochondrial antioxidant Mitoquinone (MitoQ), a compound designed to deliver ubiquinone into mitochondria, and antioxidants of SkQ-type. MitoQ has been tested successfully in human diseases such as Hepatitis C induced liver disease and skin photo damage as well as a number of experimental animal models including ischemia-reperfusion, neurodegenerative diseases, diabetes, and alcohol-induced hepatosteatosis [reviewed in 149, 150]. In addition, Rodriguez-Cuenca *et al.* [151] have demonstrated that the antioxidants targeting mitochondria can be safely administered long-term to wild-type mice.

Recently, mitochondria targeted antioxidants of SkQ-type seem to be very promising compounds aimed at eliminating mitochondrial ROS excess caused by aging. These compounds are chimeric molecules composed of penetrating lipophilic cations and plastoquinone, a powerful antioxidant and a component of the photosynthetic electron transport chain in chloroplasts of plants and in cyanobacteria, that is in structures that produce oxygen and therefore are under constant oxidative stress [reviewed in 152]. Plastoquinone derivatives, such as SkQ1 (plastoquinonyl-decyltriphenylphosphonium) and SkQR1 (plastoquinonyl-decylrhodamine 19), protect mitochondria from oxidative damage and decrease mitochondrial damage in *in vivo* models of oxidative stress [148, 153].

SkQ1 enhanced the median lifespan of organisms and retarded, arrested, and even reversed development of several age-related pathological traits. Interestingly, despite the higher dose of NAC used, the effects of SkQ1 were of the higher magnitude compared to those with NAC [154]. Since mitochondrial oxidative damage may be one of the main reasons for influenza virus-induced cell death [155] these compounds could be suggested as anti-influenza agents.

CONCLUSION

Increasing evidence has demonstrated that altered intracellular redox state occurs during influenza virus infection. These redox changes *versus* an oxidized state play a key role in the activation of numerous cell pathways that are hijacked by virus to assure its replication and/or that control inflammatory response and the fate of infected cells [3]. Then, in the last years, antioxidant therapy has been proposed to decrease viral load and to counteract lung tissue damage caused by an overproduction of ROS induced by the virus [156]. Some antioxidants are effective in this protection against infection and represent promising therapeutic molecules that could be employed in the treatment of influenza. However, other molecules caused harmful effects in experimental models or clinical trials by highlighting the "dark side" of some antioxidants. Indeed, in some cases antioxidants could act as oxidants or produce stress, if the antioxidants overcome the physiological production of reactive species [119]. Moreover, supplementation with antioxidants may provide little if any unequivocal benefit to disease prevention in humans and may potentially impair health span [157, 158]. For example, consumption of physiological amounts of vitamin C and E abrogated the capacity of physical exercise to render insulin more effective in lowering blood sugar concentrations [159, 160]. Physical exercise by generating large numbers of ROS creates the oxidative redox potential needed to oxidize the free sulphhydryl groups of cysteine into the disulphide bonds, used to stabilize the 3D conformation of physiologically active protein. Therefore, as postulated by Watson [161], an oxidative environment delays if not prevents the occurrence and severity of type 2 diabetes.

In conclusion, there are open questions that the academic community should figure out about the efficacy of each antioxidant: the dose, its actual ability to affect redox-regulated pathways as well as the redox state of the microenvironment in which it must function. Thus, all these issues should be considered in recommending their use in the therapy of influenza.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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