Glutathione (GSH), a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine) found in eukaryotic cells at millimolar concentrations, has a number of important functions in cell physiology (Meister & Anderson, 1983; Nakamura et al., 1997). In its oxidized disulphide form, GSH protects cells from oxidative stress damage by maintaining the intracellular redox state and modulating various intracellular functions via metabolic interconversion (Reed, 1986).

Several studies show an imbalance in the intracellular redox state during viral infections, accompanied by a progressive depletion of GSH and, consequently, disruption to the intracellular redox state. Recent data reported by our group have demonstrated a progressive decrease in intracellular GSH levels during parainfluenza-1 Sendai virus (SV), herpes simplex-1 virus (HSV-1) and human immunodeficiency virus (HIV) infections in vitro (Ciriolo et al., 1997; Palamara et al., 1995; Garaci et al., 1997). Several in vivo studies have also reported an imbalance in the redox state in the cells and bodily fluids of HIV- and hepatitis C-infected patients, as well as in the lungs and livers of influenza virus-infected mice (Boya et al., 1999; Elbim et al., 1999; Mileva et al., 2000). Several pieces of data suggest that the imbalance in the intracellular redox state is a key event in the development of viral replication, either by increasing viral replication (Palamara et al., 1996a) or activating nuclear transcription factors (Schreck et al., 1991). Indeed, many antioxidant substances are known to inhibit the life cycle of viruses through different mechanisms of action. Our previously published data show that the administration of reduced GSH to infected cells prevents this decrease in intracellular GSH and inhibits viral replication in SV, HSV-1 and HIV infections (Palamara et al.,...
1995; Palamara et al., 1996b; Garaci et al., 1992). In these experimental models, GSH antiviral activity appears to be related to an inhibition of the post-transcriptional stages of viral replication, probably by impeding the correct folding and maturation of specific virus envelope proteins. Other authors report that the administration of N-acetyl-cysteine, a precursor of GSH, inhibits HIV replication (Gu et al., 2001; Roederer et al., 1992) and hepatitis C replication (Gong et al., 2001) by preventing the activation of NF-kB.

The efficacy of antioxidant substances against viral infection has also been demonstrated in in vivo studies. In particular, our data report that the administration of high doses of GSH reduces viral infection and inhibits disease progression in a murine model of AIDS (Palamara et al., 1996c). Furthermore, topical GSH treatment is suggested as a therapy in HSV-induced keratitis (Nucci et al., 2000). Other authors report that administering vitamin E supplements significantly decreases pulmonary viral titre in influenza-infected old mice (Han et al., 2000).

Even though the antiviral activity of antioxidant substances has been clearly demonstrated, it has been proven that some molecules, such as GSH, are not freely transported to most cells or tissues. For this reason, we have evaluated the antiviral activity of some GSH derivatives that can more easily cross the membranes of many cell types. We investigated the effect of GSH derivatives on the replication of RNA virus (SV) and DNA virus (HSV-1) in freely permissive cells [Madin Darby canine kidney (MDCK), African green monkey kidney (Vero)]. The results reported in this paper show that GSH-C4 exerts remarkable antiviral activity in vitro against both viruses without having toxic effects on uninfected cells, suggesting its potential use in antiviral therapy.

Material and methods

Synthesis of GSH derivatives (GSH-C2, GSH-C4, GSH-C6, GSH-C8, GSH-C12)
The compounds were manually synthesized using the standard method of solid-phase peptide synthesis which follows the 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Wells & Atherton, 1997), with minor modifications.

All manual syntheses were carried out on a 0.1 mmol scale using a rotary reaction vessel with the Fmoc protected N-terminal glycine, preloaded onto Wang resin (Novabiochem AG, Laufelfingen, Switzerland).

The standard synthesis cycle involved swelling the resin in dichloromethane (Biosolve LTD, the Netherlands) for one night. Deprotection of the Fmoc group was then obtained with 20% piperidine (Fluka Chemie AG, Buchs, Switzerland) in N,N-dimethyl formamide (DMF) (Biosolve LTD, the Netherlands) for 20 min.

Five equivalents (eq.) of the appropriate Fmoc amino acid (Advanced Biotech Italia, Italy) were preactivated with 4.5 eq. of O-(benzotriazol-1-yl)-1,1,3,3 tetramethylenurnhexafluorophosphate (Advanced Biotech Italia) and five eq. of N,N diisoproplthylamine at a final concentration of 0.2 M amino acid in anhydrous N-methylpyrrolidone (Biosolve LTD, the Netherlands). This solution was coupled with in situ neutralized resin for 1 h at 40°C. As a capping step, a mixture of 5% acetic anhydride (Fluka Chemie AG, Buchs, Switzerland) in DMP after the coupling of each amino acid was included.

GSH-C4 derivative was prepared by treating peptide resin with n-butanolic acid (Fluka Chemie AG, Buchs, Switzerland), activated as previously described for amino acids. Ethanoyl, hexanoyl, octanoyl and dodecanoyl derivatives (GSH-C2, GSH-C6, GSH-C8 and GSH-C12) were similarly prepared using acetic anhydride, hexanoic, octanoic and dodecanolic acids (Fluka Chemie AG, Buchs, Switzerland) in place of butanoic acid, respectively.

Cleavage of oligomer from Wang resin and removal of the remaining side chain protecting group was performed in a single step, using trifluoroacetic acid (TFA) (Biosolve LTD, the Netherlands): ethanedithiol (Fluka Chemie AG, Buchs, Switzerland): water: triisopropylsilane (Fluka Chemie AG, Buchs, Switzerland): ethanedithiol (Fluka Chemie AG, Buchs, Switzerland) (92.5:2.5:2.5:1 v/v) for 2 h at room temperature. The resulting solution was concentrated to about 1 ml under vacuum and finally precipitated and washed in ice-cold diethyl ether.

All the molecules were purified by reverse phase high pressure liquid chromatography (RP-HPLC) with a Waters C18 μBondapack column. Solvent A was 0.1% TFA in water and solvent B was the same in acetonitrile.

The solvent program was as follows: a gradient started with 100% solvent A for 5 min and linearly increased to 60% solvent B in 30 min and up to 100% B in 5 min.

The fractions containing the relevant molecules were collected, vacuum dried and, finally, lyophilized. The molecular weights of GSH derivatives confirmed by an electrospray mass spectrometer were: 349.3, 377.3, 405.3, 433.3 and 489.3 daltons for GSH-C2, GSH-C4, GSH-C6, GSH-C8 and GSH-C12, respectively. Mass spectra of each compound were acquired in the negative ion mode using a single quadrupole HP Engine 5989-A equipped with an electrospray ion source. All products were obtained with a final yield ranging from 75–78% and a purity greater than 95% after HPLC analysis. An example of mass spectrum for GSH-C4 is shown in Figure 1.

Cells and viruses

MDCK, Vero and human histiocytic lymphoma cells (U937) were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (Flow Laboratories, Milan, Italy).
SV is a nonsegmented single-stranded RNA virus of negative polarity. Like other paramyxoviruses, SV was prepared by allantoic inoculation of embryonated eggs as previously described (Garaci et al., 1992). Confluent monolayers of MDCK cells were infected with SV [three haemagglutinating units (HAU)\(\times10^5\) cells]. After incubation for 1 h at 37°C (adsorption period), unadsorbed virus was removed. The monolayers were washed and then incubated with complete medium containing 2% fetal calf serum. Virus production was determined in the supernatants of infected cells at different times post-infection (p.i.), by measuring the HAU according to standard procedures, using human type 0 Rh+ erythrocytes. For the evaluation of antiviral activity, the compounds GSH-C2, GSH-C4 and GSH-C6 were diluted in RPMI pH 7.3 and added at the desired concentration, just after the viral adsorption period.

Clinically isolated HSV-1 TV1 was grown and titrated in Vero cells as previously described (Palamara et al., 1995). For infection, confluent monolayers of Vero cells were infected with HSV-1 at m.o.i. (multiplicity of infection) of 0.03 PFU/cell. After incubation for 1 h at 37°C (adsorption period), unadsorbed virus was removed. The monolayers were washed and then incubated with complete medium containing 2% fetal calf serum. GSH-C4 was added, at the indicated doses, just after the viral adsorption period and maintained in the culture medium until the experiments were finished. Supernatants from infected cells were harvested at different time points after virus challenge and tested for their ability to form plaques in Vero cells, using a standard titration method. Similar experiments were performed also on a thymidine-kinases-negative strain (HSV-1 TK A305), following the same procedures.

**Cell toxicity studies**

All of the compounds (GSH-C2, GSH-C4, GSH-C6, GSH-C8, GSH-C12) were studied for cytotoxicity on confluent monolayers of uninfected MDCK cells. Cytotoxicity was evaluated on the basis of microscopic examination of cell morphology, evaluation of cell viability after trypan blue staining and cell count. All the compounds were diluted in RPMI; the final pH of the solution was about 7–7.3. GSH-C2 and GSH-C4 were also studied for their eventual cytostatic effect on exponentially growing U937 cell line. To this aim, cells were stained with trypan blue and counted 24, 48 and 72 h after addition of different doses of the substances.

**Figure 1.** Molecular-chemical structure of n-butanoyl \(\alpha\)-glutamyl-cysteinyl-glycine (GSH-C4) and its MS analysis after purification

![Figure 1](image_url)

Performed in negative ion mode, the acquisition revealed a unique peak at 376.7 corresponding to \([\text{M-H}]^-\).


Enzymatic determinations

GSH peroxidase (EC 1.11.1.9.) activity in human haemolysate in the presence of GSH or its derivative GSH-C4 was measured spectrophotometrically in a system coupled with GSH reductase (1.6.4.2.) (prepared as described by Beutler, 1984). Briefly, 0.1 M Tris-HCl/0.5 mM EDTA pH 8.0, 2.0 mM GSH or GSH-C4, 1.0 U GSH reductase, 0.2 mM NADPH and 10 µl haemolysate 1:20 were added to cuvettes with a critical volume of less than 1.0 ml. Reagents were incubated at 37°C for 10 min; 0.07 mM t-butyldihydroperoxide was then added and the system's optical density (OD) decrease was measured at 340 nm.

GSH S-transferase (EC 2.5.1.18.) activity in human haemolysate, in the presence of GSH or the derivative GSH-C4 was measured spectrophotometrically as described (Beutler, 1984). Briefly, 0.1 M K₂HPO₄/KH₂PO₄ pH 6.5 and 0.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) were incubated at 37°C for 10 min, then 1.0 mM GSH or GSH-C4 and 50 µl haemolysate 1:20 were added to the mixture and the increase of OD, due to the production of CDNB-GSH conjugate, at 340 nm was measured. GSH and GSH-C4 were then oxidized by overnight incubation at room temperature in the presence of 35%/v/v H₂O₂ to obtain GSSG and C4-GSSG-C4, respectively. GSH reductase (EC 1.6.4.2.) activity was then evaluated spectrophotometrically on human haemolysate, as previously described (Beutler, 1984). Briefly, 0.05 mM Tris-HCl/0.25 mM EDTA pH 8.0, 10 µl haemolysate 1:20 and 0.1 µM FAD were incubated 10 min at 37°C, then 1.5 mM GSSG or C4-GSSG-C4 were added and the mixture was incubated again at 37°C for 10 min. Finally, 0.1 mM NADPH was added and the decrease of OD at 340 nm was evaluated.

Kinetics of GSH and GSH-C4 influx into erythrocytes

Assays of GSH and GSH-C4 influx into erythrocytes (RBCs) were performed at 37°C in 1.5 ml polypropylene micro centrifuge tubes, using ‘oil-stop’ methodology. Briefly, red blood cell suspensions at 10% haematocrit containing 5 mM GSH or GSH-C4 and 10 mM GSH were incubated 2 h at 37°C. At different times (0, 5, 15, 30, 60 and 120 min), 600 µl aliquots were stratified on 600 µl bromodocane, centrifuged 5 min at 10000 g and spectrophotometrically evaluated for GSH and GSH-C4 content in RBCs, as described (Beutler, 1984). Briefly, 30 µl RBC pellets were resuspended in 70 µl of phosphate saline solution (PBS) pH 7.4, diluted with H₂O (1:10) and 1.5 ml of precipitating solution (for composition see Beutler, 1984) was added. GSH and GSH-C4 were evaluated on clear supernatant at 412 nm after the addition of DTNB [5′,5′-dithiobis (2-nitrobenzoic acid)] reagent, a disulphide compound that is readily reduced by sulphhydryl compounds forming a highly coloured yellow anion.

GSH-C4 stability in plasma

GSH-C4 (1 mM) was incubated in human plasma at 37°C. And at different time points of incubation (0, 15, 30, 60 and 120 min), 200 µl aliquots were ultra filtered, using the Amicon Centricon 30 microconcentrators, by centrifugation at 2000 g for 30 min. The filtered solutions were directly analysed by high-performance capillary electrophoresis (HPCE) to evaluate GSH-C4 and C4-GSSG-C4 content as described (Piccoli et al., 1994).

Results

GSH derivatives toxicity and antiviral activity

The addition of C8 (n-octanoyl) and C12 (n-dodecanoyl) derivatives caused marked toxic effects and damage to uninfected MDCK monolayer cells. These effects increased dose-dependently, and were observed starting from the 0.1 mM concentration. For this reason, their activity on viral replication was not considered. The treatment of MDCK cells with GSH-C6 induced monolayer cell damage exclusively at the concentration of 10 mM. Some modification of cell morphology was detected 24 h after addition of 2.5 and 5 mM. This substance caused an inhibition of SV replication, measured as haemagglutinating activity in the supernatant, ranging between 50–100% (2.5–10 mM, respectively). Because of the limited difference between cytotoxic and antiviral doses, we did not continue the study of this derivative. GSH-C4 and GSH-C2 did not induce any toxic effect or change in morphology of cell monolayer at the concentrations used for the study (0.1–10 mM). To better investigate the eventual cytostatic effect of these compounds on exponentially growing cells, U937 cells were plated at the concentration of 2×10⁵/ml and increasing doses of GSH-C2 and GSH-C4 were added. Cell counts were performed 24–48 and 72 h after drug addition. Both compounds induced a slight, non-significant inhibition of cell proliferation compared to untreated cells when used at 10 mM concentration (10–15% decrease for GSH-C2 and GSH-C4, respectively). No differences were detected after addition of both compounds at 1, 2, 2.5, 5 and 7.5 mM concentrations.

Regarding the antiviral activity of GSH-C2, its addition to SV infected MDCK cells caused a slight reduction of viral replication. The viral titre detected in the supernatant of cells treated with 5.0 and 7.5 mM GSH-C2 was decreased by 30 and 40%, respectively, compared to untreated infected cells. Furthermore, GSH-C2 had no effect on HSV-1 replication. For these reasons, the study was then finalized in order to better characterize GSH-C4 activities.

GSH-C4 metabolism

The enzymatic activities of GSH peroxidase and GSH S-transferase from human haemolysate were evaluated in the presence of either 2 mM GSH-C4 or GSH as substrates.
GSH peroxidase and GSH S-transferase activities in the presence of GSH-C4 were 1.8% and 3.5% compared to GSH, respectively. Moreover, GSH and GSH-C4 in the corresponding disulphide forms were chemically oxidized. GSH reductase activity on both substrates was then evaluated. The results obtained show that C4-GSSG-C4 is slowly reduced by the enzyme, having an activity of 0.63 IU/g haemoglobin (Hb), a Km of 50 mM and a Vmax of 12.6 µmol/min/mg Hb compared to an activity of 3.0 IU/g Hb, a Km of 1.3 mM and a Vmax of 6.3 µmol/min/mg Hb obtained with GSSG.

GSH-C4 influx into erythrocytes
The influx of GSH-C4 into erythrocytes was evaluated and compared to GSH. The results obtained show that GSH-C4 permeates the erythrocyte membrane more quickly than GSH; in fact, the rate of influx (calculated in the first 30 min) was 4.33 nmol/min/ml RBCs for GSH-C4 and 2.33 nmol/min/ml RBCs for GSH.

GSH-C4 stability in plasma
The ability of human plasma enzymes to convert GSH-C4 in C4-GSSG-C4 was also evaluated. As shown in Figure 2, plasma enzymes are able to oxidize GSH-C4 with the stoichiometric production of C4-GSSG-C4 (t1/2 50 min).

Effect of GSH-C4 on SV replication
GSH-C4 inhibits replication of SV in MDCK cells in a dose dependent manner (Figure 3). An inhibition ranging between 88% (24 h p.i.) and 93% (48 h p.i.) was found in the presence of 5 mM GSH-C4. No virus was detected in the supernatant of cells treated with 7.5 mM of the drug. The optimal antiviral dose of GSH-C4 (7.5 mM) was not toxic for the cells; this was confirmed by microscopic examination of the monolayers and by vital dye exclusion. The 50% inhibition of viral production (EC50) at 48 h p.i. was obtained at the concentration of 3.6 mM; 7.5 mM of GSH was necessary to obtain the same results. It is noteworthy that in different experimental models (Garaci et al., 1992; Palamara et al., 1995; Kalebic et al., 1991; Vogel et al., 2003) the GSH antiviral activity has been described in doses ranging between 1–15 mM.

At the concentrations used in this study, GSH-C4 did not induce toxic effects on uninfected cells. This was confirmed by microscopic examination of the monolayers and by vital dye exclusion (not shown).

Effect of GSH-C4 on HSV-1 replication
The effect of different doses of GSH-C4 on HSV-1 production in Vero cells is shown in Figure 4; data represent the viral titre in the supernatant of infected cells 48 h after infection. The results obtained show that GSH-C4 inhibited virus replication in a dose-dependent manner. A marked decrease (approx. 3 log10) in viral replication was achieved through the addition of 7.5 mM GSH-C4. No virus particles were detected at 10 mM. The same inhibition was found 72 h after infection (not shown).

Figure 2. GSH-C4 stability in plasma

GSH-C4 (1 µmol/ml plasma) was incubated for 2 h at 37°C. Ultrafiltered samples were prepared at different incubation times and analysed by high-performance capillary electrophoresis (HPCE). Values are the mean ± SD of three different experiments.
The addition of GSH at the concentration of 10 mM did not induce a complete inhibition of the virus, and produced only a 2.5 log₁₀ reduction of virus replication (Figure 4). Furthermore, unlike GSH, GSH-C₄ (7.5 mM) markedly reduced the typical cytopathic effect induced by HSV-1 (data not shown).

A percentage of HSV strains (approx. 5% in immunocompromised and <1% in immunocompetent patients) has become resistant to conventional therapy (Field, 2001), such as acyclovir, as a consequence of a mutation in the thymidine-kinase gene (TK⁻). For this reason, we also tested the effect of GSH-C₄ on a TK⁻ strain. The results obtained (Table 1) show that GSH-C₄ is less active against the TK⁻ strain than the wild type strain. In fact, a significant inhibition of virus titre (about 1 log₁₀) was observed only at a concentration of 7.5 mM 72 h p.i. However, the HSV-1 TK⁻ strain used in this study was a deletion clone (Δ305), with a lower replicative rate (about 3 log₁₀) than the parental strain. Thus, the lower sensitivity of this HSV-1 TK⁻ strain to inhibition by GSH-C₄ could not be considered representative of all TK⁻ strains isolated from clinical samples that usually replicate normally in tissue cultures.

**Discussion**

GSH probably exerts its antimicrobial activity in a number of different ways; for example, by controlling viral or microbial growth by affecting the correct folding of essential pathogen proteins [see Palamara et al., 1996b (HIV-1 Gp 120); Nencioni et al., 2003, Cai et al., 2003 (influenza A...
Antiviral activities of glutathione derivatives

haemoagglutinin), interfering with the activation of selected transcription factors [Roederer et al., 1990; Mihm et al., 1995 (NF-κB)] and affecting viral receptor expression (Papi et al., 2002) or function (Matthias et al., 2002). Thus, GSH can be considered an antimicrobial agent that exerts its activity through several different mechanisms, depending on the host-pathogen systems considered and on the GSH concentration applied. Unfortunately, GSH in vivo is rapidly oxidized, particularly in the presence of viral infections when the redox state of the cells is unbalanced by a massive production of reactive oxygen species. Oxidized GSH is in turn reduced by cellular GSH reductases or pumped out of the cell by an ATP-dependent mechanism. GSH reduction by cellular enzymes depends on the availability of cell-reducing equivalents (NADH, NADPH); this is not favourable in cases of pathogenic infection. Instead, the expulsion of oxidized GSH by the cell can only be counterbalanced by new intracellular synthesis of the tripeptide. GSH reduction by cellular enzymes depends on the availability of cell-reducing equivalents (NADH, NADPH); this is not favourable in cases of pathogenic infection. Instead, the expulsion of oxidized GSH by the cell can only be counterbalanced by new intracellular synthesis of the tripeptide. Taking into account these considerations, we deduced the possibility of using GSH derivatives capable of crossing biological membranes in the treatment of several viral infections. The molecules reported in this paper represent a first class of such GSH derivatives. The addition of aliphatic chains at the α-NH₂ of glutamic acid represents, in effect, a useful approach in the production of diffusible drugs because of the hydrophobic properties of the chain and the neutralization of a charged NH₂ group. Unfortunately, aliphatic chains longer than four C atoms are toxic to the cells, while a single ethanoyl group show no or little effect. Thus, the butanoyl derivative of GSH was extensively characterized. It is important to note that, like GSH, this GSH derivative could act via different mechanisms. It probably interferes with different phases of the viral life-cycle, since it is able to prevent the cytopathic effect of HSV-1 in Vero cells but also inhibits SV replication in MDCK-infected cells. Understanding butanoyl GSH’s mechanism of action is beyond the scope of this paper but certainly will be further investigated in our laboratories. The fact that butanoyl GSH can probably be used not only as a water-soluble drug but also in the form of a cream or lotion, particularly in the inhibition of HSV-1 cytopathic effect by local applications, has not escaped our attention.

The GSH derivatives we have designed, synthesized and characterized are new but other derivatives have been previously reported. Meister and co-workers reported almost 20 years ago on the preparation and characterization of GSH monoethyl ester (Puri & Meister, 1983; Anderson et al., 1985; Wellner et al., 1984; Anderson et al., 1989; Naganuma et al., 1990), a derivative that is intracellularly transported and split into GSH. Burg et al. (2002a) have reported on the synthesis of peptidomimetic GSH-conjugate analogues that are effective inhibitors of multidrug resistance protein 1 (MRP1) and thus can have a role in anticancer drug resistance (Burg & Mulder, 2002b).

Figure 4. Effect of GSH-C4 on HSV-1 replication

Vero cells were infected for 1 h with HSV-1 at m.o.i. of 0.03 PFU/cell. After extensive washings, GSH-C4 at different concentrations (0–10 mM range) or 10 mM GSH were added to cell cultures. Virus replication was assayed in Vero cells 48 h post infection by using the plaque assay. Each point represents the average ± SD of quadruplicate samples. Data are from a single experiment representative of 4.

*P<0.05, ***P<0.0001.
Finally, nitric oxide derivatives (S-nitrosoglutathione) have been extensively investigated as antioxidants (Rigobello et al., 2002) and nitric oxide donors (Kuo et al., 2003). These last derivatives, as well as a number of GSH conjugates, can also occur in vivo and represent a useful system for the transport of chemically reactive metabolites of both autologous and exogenous origins (Baillie & Slatter, 1991). Many GSH derivatives are formed through the SH reactive group of this peptide; however, usually these compounds are unstable because of the action of endogenous GSH metabolizing enzymes. Butanoyl GSH is stable but can be oxidized as free GSH. Once oxidized in dimeric form, the cellular GSH reductase is not able to efficiently reduce it back to butanoyl GSH. Thus, butanoyl GSH is not a good substrate for GSH metabolizing enzymes and exerts its primary effect as a reducing agent, with a plasma half-life of about 50 min (see Figure 2), while free GSH has a half-life of 30 min (Magnani et al., 1984). The results reported in this paper and in the available literature suggest that butanoyl GSH can be considered an interesting antimicrobial agent with proven efficacy in vitro against different pathogens, with a potential place among compounds that can reduce both viral infectivity in early disease and viral production in later disease. This property is nearly unique and worth further exploitation.

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References


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