Inhibitory effect of thiosemicarbazone derivatives on Junin virus replication in vitro

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The inhibitory effect of several thiosemicarbazones (TSCs), synthesized from aromatic ketones and terpenones, and their heterocyclic thiazolines (TDZ) derivatives, was investigated against Junin virus (JUNV), an arenavirus agent of Argentine haemorrhagic fever. From the 25 compounds tested, six compounds belonging to the TSC group were found to be selective inhibitors of JUNV, with EC50 values determined by a virus yield inhibition assay in the range 3.4–12.5 µM, and selectivity indices greater than 10. By contrast, most of the TDZs obtained by heterocyclization of the TSCs were not active against JUNV. No conclusive structure–activity relationships could be established but systematically higher activity was associated to TSCs derived from aromatic ketones.

The mode of action of one of the most active compound, the 3,4-dihydronaphthalen-1(2H)-one thiosemicarbazone (tetralone thiosemicarbazone), was studied further. This TSC lacked virucidal effects on JUNV virions. Results from time of addition experiments and viral protein expression assays suggest that tetralone thiosemicarbazone inhibited a late stage in the replicative cycle of JUNV.

Keywords: thiosemicarbazone derivatives, antiviral, Junin virus, hemorrhagic fever, arenavirus

Introduction

A considerable number of thiosemicarbazone (TSC) derivatives has been reported as antibacterial, antiviral and antiproliferative compounds. In particular, certain TSCs showed a selective inhibition of herpes simplex virus (HSV) infection in vitro, based on inactivation of the viral enzyme ribonucleotide reductase (Blumenkopf et al., 1992; Prichard & Shipman, 1995), and also TSCs were active inhibitors of HSV genital infection in guinea pigs and mice in vivo (Sidwell et al., 1990). The effect of TSCs against human immunodeficiency virus (HIV) was also reported to target the structural protein synthesis of HIV (Teitz et al., 1994). In contrast, thiazolines (TDZs), compounds obtained by heterocyclization of TSCs, were screened only for their antibacterial activity (Ali & Alam, 1994), but their antiviral properties have not been investigated.

In this study, we report the inhibitory activity of several TSCs, synthesized from aromatic ketones and terpenones, and their heterocyclic TDZ derivatives, against Junin virus (JUNV), an RNA virus member of the Arenaviridae and agent of Argentine hemorrhagic fever (AHF) (Damonte, 2002). AHF is an endemic/epidemic disease recognized as a major public health problem in certain agricultural zones of Argentina. The current therapy for AHF patients is the early administration of standardized doses of convalescent plasma, but this therapy is not effective when initiated after a week of illness and 10% of treated patients develop late neurological complications (Enria & Maiztegui, 1994). The antiviral activity of diverse types of compound against JUNV infection in vitro has been reported (Andreis & De Clercq, 1990; Candurra et al., 1996; Castilla et al., 1998; García et al., 2000; Damonte & Coto, 2002). However, ribavirin is the only compound that has shown partial efficacy against JUNV infection in vivo, but with a high level of undesirable secondary reactions (McKee et al., 1988; Enría & Maiztegui, 1994). Thus, there is a real need for active drugs against JUNV and other viruses that produce hemorrhagic disease in humans (Andreis & De Clercq, 1993).

Materials and methods

Chemistry

The structures of the tested compounds are shown in Figure 1. The TSCs 1a to 7a (Martins Alho et al., 2000), 8a (Somogyi, 1991) and 9a to 12a (Brousse et al. 2002)
were obtained from the corresponding ketones and terpenones by treatment with thiosemicarbazide (Figure 2). In particular, the compound 3,4-dihydronaphthalen-1(2H)one thiosemicarbazone, named 4a, was obtained from equimolecular quantity of 2,3,4-trihydronaphthalen-1-one and thiosemicarbazide by the general procedure (Martins Alho
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et al., 2000). The TDZs 1b, 4b, 6b and 7b (Martins Alho et al., 2000), and 9b to 11b (Bousseau et al., 2002) were obtained by cyclization of the corresponding TSCs (Figure 2).

Melting points were uncorrected. 1H-NMR and 13C-NMR spectra were recorded on a Bruker 400 MHz instrument in the indicated deuterated solvent with chemical shifts reported in parts per million (ppm). FTIR was performed on a Nicolet IR spectrophotometer and mass spectra obtained were recorded on a spectrometer VG AutoSpec (Micromass Inst.). TLC analyses were performed on an analytical thin plates coated with Silica gel 60 F254 (Merck) and components were visualized under UV. All commercial reagents and solvents were used without purification.

5-Methyl-2-(1-methylethyliden)cyclohexanone thiosemicarbazone (13a)

Title compound was prepared from the equimolecular quantity of pulegone and thiosemicarbazide dissolved in ethanol. The mixture was heated at reflux, monitoring the reaction by TLC. Evaporation under reduced pressure gave the thiosemicarbazone. This compound was purified by p-TLC, solvent Cl2CH2. The thiosemicarbazone 13a was obtained as oil. Yield 87%.

1H-RMN: (CDCl3), δ: 0.93 (3H, d, J=6.14), 1.12 (1H, m), 1.56 (3H, s), 1.70 (3H, s), 1.71–1.75 (3H, m), 2.20 (1H, m), 2.40 (1H, m), 2.69 (1H, m), 6.62 (1H, NH, s), 7.17 (1H, NH, s), 8.49 (1H, NH, s); 13C RMN (CDCl3) δ: 19.8 (CH3), 21.7 (CH3), 22.4 (CH3), 30.1 (CH3), 35.2 (CH2), 44.4 (CH2), 126.2 (C=C), 131.0 (C=C), 156.1 (C=N), 177.7 (C=S); IR (film) 3430, 3364, 3261 (NH), 1589 (C=S), 1484 (C=S), 1484 (C-N) cm-1; MS CI+ m/e 226.13718 (M+H)+, calculated 226.13779.

General procedure for the synthesis of 5-amine-3-N-acetyl-2,2-disubstituted-1,3,4-thiadiazoline

A mixture of thiadiazoline (11.25 mmol) and 36 ml of hydrazine hydrate (99%) was stirred at room temperature for 3 h. The resulting precipitate was collected by filtration and recrystallized from water to give 5-amine-3-N-acetyl-2,2-disubstituted-1,3,4-thiadiazoline.

5-Amine-3-N-acetyl-2,2-diphenyl-1,3,4-thiadiazoline (1c)

Yield 81%, mp: 125.5–127.5°C (water).

1H RMN (CDCl3), δ: 2.25 (3H, s), 4.25 (2H, bs), 7.25–7.55 (10 H, complex absorption); 13C RMN (DMSO-d6), δ: 24.0 (COCH3), 88.3 (C2), 127.5, 127.6, 127.9, 148.8 (C5), 166.0 (C=O) ppm; IR (film) 3430, 3319 (NH), 1627 (C=O), 1412 (C-N) cm-1; MS CI+ m/e 297.09422, calculated 297.09428.

Spiro-[5-amine-3-N-acetyl-2,1′,2′,3′,4′-tetrahydronaphthalen]-1,3,4-thiadiazoline (4c)


1H RMN (CDCl3), δ: 1.75–1.90 (1H, cdd, J1=J2=J3=12.0 Hz, J4=5.0 Hz, J5=2.5 Hz), 2.05–2.15 (1H, m), 2.24 (3H, s), 2.40–2.48 (1H, m), 2.75–2.85 (1H, m), 2.90–3.00 (1H, m), 3.05–3.11 (1H, td, J1=J2=13.3 Hz, J3=3.1 Hz), 4.20 (2H, bs), 7.05 (1H, d, J=7.60 Hz), 7.13–7.20 (2H, m), 7.55 (1 H, d, J= 7.60 Hz); 13C RMN (CDCl3), δ: 22.5, 24.9, 29.5 (C3′, C4′, CH3), 37.0 (C1), 87.5 (C1′), 127.3, 127.3, 128.4, 129.5 (C5′, 6′, 7′, 8′), 136.4 and 137.8 (C4′a, C8′a), 148.1 (C-N), 168.1 (C=O); IR (film) 3313, 3194 (N-H), 1620 (C=O), 1413 (C=N) cm-1; MS CI+ m/e 261.09328, calculated 261.09358.

Spiro-[5-amine-3-N-acetyl-2,2′-(1′,3′,3′-trimethylbicycle[2,2,1]-heptane)]-1,3,4-thiadiazoline (10c)


1H RMN (CDCl3), δ: 0.97 (3H, d, J=11.0 Hz), 1.29 (3H, s), 1.45–1.47 (1H, m), 1.55–1.65 (3H, m), 1.87 (1H, complex absorption), 2.22 (1H, s), 3.18 (1H, dd, J=11.0 Hz, J=2=5.0 Hz), 4.20 (2H, bs), 7.05 (1H, d, J=7.60 Hz), 7.13–7.20 (2H, m), 7.55 (1 H, d, J= 7.60 Hz); 13C RMN (CDCl3), δ: 17.8 (C10′), 23.5 (C6′), 25.5 (COCH3), 26.5 (C9′), 26.8 (C8′), 41.4 (C5′), 42.8 (C7′), 49.4 (C4′), 52.2 (C1′), 52.3 (C3′a), 106.1 (C2′), 150.8 (C5), 168.6 (C=O) ppm; IR (film) 3131, 3180 (N-H), 1620 (C=O), 1413 (C-N) cm-1; MS CI+ m/e 267.13955, calculated 267.13958.
Virology

Cells and viruses

Vero cells were grown as monolayers in Eagle’s minimum essential medium (MEM, GIBCO, USA) containing 5% inactivated calf serum and 50 µg/ml gentamicin. Maintenance medium (MM) consisted of MEM supplemented with 1.5% calf serum and gentamicin.

The naturally attenuated IV4445 strain of JUNV obtained from a mild human case and the TRIV 11573 strain of Tacaribe virus were used. Virus stocks were prepared in Vero cell cultures and titrated by plaque formation.

Cytotoxicity assay

Cytotoxicity was measured with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, USA] method in Vero cells. Confluent cultures of stationary Vero cells grown in 96-well plates were exposed to twofold dilutions of the compounds ranging 200–3.12 µM in MM, with three wells for each dilution, during 48 h of incubation at 37°C. Then, 10 µl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation, the supernatant was decanted and 200 µl ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader and cytotoxicity was calculated as the cytotoxic concentration 50% (CC50), compared to controls.

To measure the effects of 4a on proliferating cultures, Vero cells were seeded in 96-well plates at a density of 1.5x10⁴ cells/well and incubated in growth medium at 37°C. Twofold dilutions of 4a (range 200–3.12 µM) were added 5 h after seeding in growth medium. After 48 h of incubation, MTT assay was performed as described above.

Virus yield inhibition assay

Vero cells grown in 24-well microplates were infected at a multiplicity of infection (m.o.i.) of 0.1. After 1 h adsorption at 37°C, cells were washed and re-fed with MM containing different concentrations of the compounds in the range of 100–1.5 µM (two wells per concentration). Positive virus controls without compound were performed simultaneously. After 48 h of incubation at 37°C, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The effective concentration 50% (EC50) was calculated as the concentration required to reduce virus yield by 50% in the compound-treated cultures compared with untreated ones by plaque formation in Vero cells.

Virion inactivation assay

Equal volumes of a virus suspension containing approximately 3x10⁸ PFU of JUNV and various concentrations of compound in MM, ranging 100–1.5 µM, were mixed and incubated for 1.5 h at 37°C. A virus control was also performed by incubation of the virus suspension with MM under the same conditions. Then, mixtures were chilled and diluted further with MM before being placed on Vero cell cultures for plaque assay.

Time of addition experiment

Vero cells grown in 24-well plates were allowed to adsorb JUNV at a m.o.i. of 0.1 for 60 min at 4°C. After removal of the inocula, the cells were washed twice with PBS and then MM containing 50 µM of the compound was added to infected cells at various times after infection and further incubated at 37°C. In all cases, extracellular virus yields were measured at 24 h post-infection (p.i.).

Assay for viral protein synthesis

Vero cells were infected with JUNV at a m.o.i. of 1. At 44 h p.i. infected cells were incubated in methionine-cysteine-free medium for 1.5 h and then labelled with 100 µCi/ml EXPRE³S³S (NEN Dupont, USA) for 3.5 h. This time p.i. was chosen to study the effect of 4a on synthesis of viral proteins, because it is the point corresponding to maximal detection of JUNV proteins (Candurra et al., 1990). The compound 4a (50 µM) was added with the methionine-cysteine-free medium and maintained during the labelling period. After labelling, cells were washed three times in cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 0.4 mM phenylmethylsulphonyl fluoride and 1% sodium deoxycholate in 0.01 M Tris-HCl pH 7.4. Samples of the clarified cell lysates were mixed with polyclonal rabbit anti-JUNV serum and incubated for 30 min at 37°C and 90 min at 4°C. Antibody–antigen complexes were collected with protein A-sepharose, washed three times in RIPA buffer and solubilized by boiling for 2 min in sample buffer containing 5% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.005% bromphenol blue in 0.0625 M Tris-HCl, pH 6.8. Viral polypeptides were then electrophoresed on 12% SDS-polyacrylamide gels and visualized by fluorography.

Results

Screening of compounds for cytotoxicity and antiviral activity

The toxicity of all compounds for Vero cells was first investigated by assessing their effects on stationary cell viability. According to compound solubility, the highest concentration assayed was 200 µM and the values of CC50 varied in...
the range 36.1 to >200 µM (Table 1). Then, the antiviral activity was examined at concentrations below the corresponding CC50 for each compound. As can be seen in Table 1, only three TSC derivatives (2a, 3a and 13a) were totally inactive against JUNV and most of the remaining ten TSCs were able to inhibit JUNV replication with EC50 values in the range 3.4–100 µM. Among these active TSCs, six compounds (1a, 4a, 6a, 8a, 10a and 11a) were the most selective inhibitors, exhibiting selectivity indices (SI), defined as the CC50/EC50 ratio, greater than 10.

By contrast, most of the seven TDZs obtained by heterocyclization of the TSCs, were not active against JUNV, with the only exception of 4b that showed a very weak antiviral effect and 9b that showed a good activity (Table 1).

Given the observed differences between TSC and TDZ, in an attempt to establish structure–activity relationships, two by-products obtained in the synthesis of the compound 4b, the products 14 and 15 (Figure 1) were evaluated for their inhibitory effect against JUNV. Although these two compounds presented a structure related to the TSCs, both were totally unable to inhibit JUNV, with EC50 values >200 µM. These data suggested that the activity could be due to the presence of the unsubstituted NH2 in the TSCs and not to the C=N bond. To test this hypothesis, the NH2-linked to C-5 in the heterocycle of three TDZs, the weakly active 4b and the inactive compounds 1b and 10b, was unprotected. Among the three new compounds obtained, named 1c, 4c and 10c (Figure 1), only 1c showed a very slight increase in antiviral potential, whereas 4c and 10c maintained the properties of the original TDZs (Table 1).

Mode of action of compound 4a
The compound 4a, TSC of tetralone, with a SI of 14.9, was chosen for further characterization of the mode of action of this kind of compounds. First, the lack of effect of this compound on replicating cells was analysed. To this end, the cytopathicity assay was performed by addition of the compound on logarithmically growing cells, at 5 h after seeding. The value of CC50 obtained under these conditions, after 48 h of incubation with compound 4a, was 76.3 µM, comparable to the CC50 obtained in stationary cells (87 µM). Tacaribe virus, other arenavirus closely related to JUNV, was similarly susceptible to 4a showing an EC50 value of 9.8 µM. In addition, the two reagents used to synthesize 4a, the thiosemicarbazide and the tetralone, were inactive against both arenaviruses (EC50 >200 µM), indicating that the structure of the whole molecule is required for the antiviral action.

To test whether 4a had direct virion inactivating properties, a virucidal assay was performed. When a JUNV suspension was incubated at 37°C for 1.5 h with either different concentrations of 4a or MM, and then remaining infectivity was titrated, no differences in virus titres were found between compound-treated and untreated virus suspensions (data not shown). Thus, 4a did not exert direct virucidal effect but had a true antiviral activity on JUNV infection of Yero cells.

To determine the time in the JUNV replication cycle at which 4a caused its inhibitory action, the effect of the time of addition of the compound on virus yield was next examined. As shown in Figure 3, a similar level of inhibition was observed if 50 µM 4a was added immediately after virus adsorption (time 0) or as late as 12 h post-adsorption. A significant level of virus yield inhibition was still observed when compound was added at 15–18 h p.i. The replicative cycle of JUNV lasts approximately 12 h until infectious progeny is released from the cell (Mersich et al., 1981). However, enough virus production for an antiviral yield inhibition assay is only detected at 24 hours post-infection.
6

3

12

15

2

18

9

tion. Results are expressed as percent of virus yield in indicated times. Virus yields were determined after 24 h of infection (50 µM) was added immediately after adsorption (time 0) or at the indicated times. Virus yields were determined after 24 h of infection. Results are expressed as percent of virus yield in 4a-treated cultures with respect to untreated ones. Values are the mean value of duplicate determinations.

blumenkopf and HIV-1 have been reported (sidwell 1990; teitz et al., 1992; prichard & shipman, 1995), whereas in other studies the cytotoxic effects of TSCs were more prominent than their antiviral properties. No conclusive structure–activity relationships could be established but systematically higher activity was associated to TSCs derived from aromatic ketones, characterized by a planar double-bond C=N in the aromatic ring. The dissimilar antiviral activity observed coming from terpenone derivatives (for example, by comparison of 10a with 9a and 11a with 13a, as well as the high activity shown by 9b) could be attributed to factors suited to the topology of the molecules rather than to the functional group TSC or TDZ.

The mode of action of one of the most active compounds, 4a, was partially characterized. This compound did not inhibit virucidal properties, and in timing studies it was found that virus yield was inhibited at any time the compound was added after virus infection. In fact, the expression of viral proteins in infected cells was not impaired by this TSC. These data could imply that an event occurring very late in infection such as virion assembly or egress could be the target in the viral cycle. In addition, the inhibitory properties were not restricted to JUNV, also other arenavirus such as Tacaribe virus was similarly susceptible to the action of 4a.

Unlike other TSCs, these compounds did not inhibit significantly HSV (data not shown) presenting a more restricted spectrum of antiviral action. This may be responsible for the higher selectivity shown by some products of this novel series of derivatives in comparison with other TSCs, as mentioned above.

Viral haemorrhagic fevers still represent a challenge in the field of antiviral chemotherapy (Andre & De Clercq, 1993). Ribavirin is the only antiviral drug that is known to inhibit JUNV replication in Vero cells.

Vero cells were infected with JUNV (m.o.i. 0.1) and compound 4a (50 µM) was added immediately after adsorption (time 0) or at the indicated times. Virus yields were determined after 24 h of infection. Results are expressed as percent of virus yield in 4a-treated cultures with respect to untreated ones. Values are the mean value of duplicate determinations.

Figure 3. Time of addition experiment

Vero cells were infected with JUNV (m.o.i. 0.1) and compound 4a (50 µM) was added immediately after adsorption (time 0) or at the indicated times. Virus yields were determined after 24 h of infection. Results are expressed as percent of virus yield in 4a-treated cultures with respect to untreated ones. Values are the mean value of duplicate determinations.

Discussion

Diverse types of TSC derivatives have previously been investigated for antiviral activity with variable success. Selective and moderately potent inhibitors against HSV-1 and HIV-1 have been reported (sidwell et al., 1990; blumenkopf et al., 1992; teitz et al., 1994; prichard & shipman, 1995), whereas in other studies the cytotoxic effects of TSCs were more prominent than their antiviral properties and no specific action could be demonstrated at the non-toxic concentrations (eason et al., 1992; kolocouris et al., 2002). In the present study, the antiviral activity of a novel series of TSCs and the corresponding TDZs, derived by heterocyclization, was investigated against the arenavirus JUNV. The results obtained showed that from the 25 compounds tested, six compounds belonging to the TSC group (1a, 4a, 6a, 8a, 10a and 11a) were found to have a selective antiviral effect, with SI values over 10, whereas only the 9b TDZ was effective to inhibit JUNV replication in Vero cells.

The mode of action of one of the most active compounds, 4a, was partially characterized. This compound did not exhibit virucidal properties, and in timing studies it was found that virus yield was inhibited at any time the compound was added after virus infection. In fact, the expression of viral proteins in infected cells was not impaired by this TSC. These data could imply that an event occurring very late in infection such as virion assembly or egress could be the target in the viral cycle. In addition, the inhibitory properties were not restricted to JUNV, also other arenavirus such as Tacaribe virus was similarly susceptible to the action of 4a.

Unlike other TSCs, these compounds did not inhibit significantly HSV (data not shown) presenting a more restricted spectrum of antiviral action. This may be responsible for the higher selectivity shown by some products of this novel series of derivatives in comparison with other TSCs, as mentioned above.

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Figure 4. Synthesis of viral proteins

Vero cells were infected with JUNV and at 44 h p.i. cells were incubated in methionine-cysteine-free medium for 1.5 h and then labelled with 100 µCi/m of EXPRE35S-35S for 3.5 h. The compound 4a (50 µM) was added with the methionine-cysteine-free medium and maintained during labelling period. Viral polypeptides were immunoprecipitated from cell lysates with anti-JUNV serum and analyzed by electrophoresis. Lane 1, mock infection; lane 2, virus control; lane 3, 4a treatment. The molecular mass markers are indicated in the left, arrows in the right indicate the positions of the main viral polypeptides.
The study showed a level of their therapeutic potential against arenaviruses. The most potent TSC derivatives evaluated in the present study showed a level of in vitro selectivity against JUNV comparable to that of ribavirin (Table 1), and consequently it is a promising lead compound to pursue further analysis of other chemically modified derivatives to establish their therapeutic potential against arenaviruses.

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