Indomethacin has a potent antiviral activity against SARS coronavirus

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Severe acute respiratory syndrome (SARS) is a newly emerging, highly transmissible and fatal disease caused by a previously unknown coronavirus (SARS-CoV). Existing in non-identified animal reservoirs, SARS-CoV continues to represent a threat to humans because there is no effective specific antiviral therapy for coronavirus infections.

Objectives: Starting from the observation that cyclopentenone cyclooxygenase (COX) metabolites are active against several RNA viruses, we investigated the effect of the COX inhibitor indomethacin on coronavirus replication.

Methods: Work involving infectious SARS-CoV was performed in biosafety level 3 facilities. SARS-CoV was grown in monkey VERO cells and human lung epithelial A549 cells, while canine coronavirus (CCoV) was grown in A72 canine cells. Antiviral activity was analysed by determining infective virus titres by $TCID_{50}$, viral RNA synthesis by Northern blot analysis and real-time RT-PCR, and viral protein synthesis by SDS-PAGE analysis after ^{35}S-methionine-labelling. Antiviral efficacy *in vivo* was determined by evaluating virus titres in CCoV-infected dogs treated orally with 1 mg/kg body weight indomethacin (INDO).

Results: Unexpectedly, we found that INDO has a potent direct antiviral activity against the coronaviruses SARS-CoV and CCoV. INDO does not affect coronavirus binding or entry into host cells, but acts by blocking viral RNA synthesis at cytoprotective doses. This effect is independent of cyclooxygenase inhibition. INDO's potent antiviral activity (>1,000-fold reduction in virus yield) was confirmed *in vivo* in CCoV-infected dogs.

Conclusions: The results identify INDO as a potent inhibitor of coronavirus replication and suggest that, having both anti-inflammatory and antiviral activity, INDO could be beneficial in SARS therapy.

Introduction

Severe acute respiratory syndrome (SARS) is a new disease with a high fatality rate that emerged in Southern China in late 2002. Within a remarkably short period of time following the worldwide epidemic that caused over 780 fatalities in 2002–2003, the SARS causative agent was identified as a novel highly infectious coronavirus named SARS-CoV [1,2]. Coronaviruses are enveloped, positive-strand RNA viruses highly diffused among humans and mammals and commonly associated with respiratory or enteric infections [3]. SARS-CoV infection causes an unusually severe febrile illness, with myalgia, headaches and respiratory symptoms, followed by progression to acute respiratory distress and respiratory failure [4].

Although the SARS epidemic has been controlled by conventional measures, the animal reservoir of SARS-CoV has not been identified and sporadic cases continue to arise in Southern China, possibly because of human contact with the animal host [4,5]. The possibility that the virus could be reintroduced into the human population is therefore likely, and effective antiviral drugs against coronaviruses are urgently needed.

We have shown that cyclopentenone prostaglandins have a potent antiviral activity against several RNA viruses by interfering with stress-sensitive cellular signal transduction pathways and transcription factors, including the heat shock factor type-1 and nuclear factor-κB [6–9]. During experiments aimed at identifying cyclooxygenase (COX) metabolites active against coronaviruses in a model of canine coronavirus (CCoV) infection, we utilized the COX inhibitors indomethacin (INDO) and aspirin as negative controls.

In the present report we describe how, unexpectedly, INDO was found to possess a potent antiviral activity against CCoV, being able to dramatically inhibit virus replication and protect the host cell from virus-induced damage. A remarkable antiviral activity was also found *in vivo* in the natural host. We then found that the

antiviral activity is not limited to CCoV and that INDO is also very effective against human SARS-CoV.

Materials and methods

Cell culture and treatment

Human A549 alveolar type II-like epithelial cells and African green monkey kidney VERO E6 cells were obtained from LGCPromochem-ATCC (Milan, Italy). Canine adenocarcinoma (A72) cells were obtained from Dr M Ferrari (Centro Substrati Cellulari, Istituto Zooprofilattico Sperimentale della Lombardia e Emilia-Romagna, Brescia, Italy). VERO, A72 and A549 cells were grown at 37°C in a 5% CO_2 humidified atmosphere in Modified Eagle Medium (MEM) (VERO cells), DMEM medium (A72 cells) or F-12K medium (A549) (GIBCO) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics (complete medium). Indomethacin (Sigma Aldrich, Milan, Italy) was dissolved in absolute ethanol at a concentration of 40 mM and diluted in culture medium immediately before use. Unless otherwise specified, INDO was added immediately after the 1 h adsorption period and maintained in the medium for the duration of the experiment. Controls received equal amounts of ethanol diluent, which did not affect cell viability or virus replication (data not shown). Aspirin and ribavirin (RBV) (Sigma), as well as interferon α (IFN-α) (Hu-rIFNα, Intron A; Schering-Plough, Milan, Italy) were added immediately after the 1 h adsorption period and maintained in the medium for the duration of the experiment. Controls received equal amounts of appropriate diluent. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to MTT formazan conversion assay (Sigma), as described [10].

Coronavirus infection and titration

Canine CoV (CCoV, strain S-378) and SARS-CoV (TOR-2 isolate, kindly provided by Heinz Feldmann, National Microbiology Laboratory,Winnipeg, Canada) were prepared by infecting A72 and VERO cell cultures, respectively. The maintenance medium consisted of MEM without FCS. Virus yields were determined by plaque forming units (PFU) and 50% tissue culture infective dose ($TCID_{50}$) titrations in confluent cells as previously described [11,12], and virus stocks were stored at –80°C. For *in vitro* infection, confluent cell monolayers were infected with CCoV or SARS-CoV for 1 h at 37°C at a multiplicity of infecion (MOI) of 10 PFU/cell, unless otherwise stated. After the adsorption period, the viral inoculum was removed and cell monolayers were washed three times with PBS and incubated at 37°C with medium without FCS for CCoV or with 2% FCS for SARS-CoV. Virus yields were determined by $TCID_{50}$ assay. All work involving infectious SARS-CoV was performed in a biosafety level 3 facility in accordance with World Health Organization recommendations.

Animal maintenance and infection

A group of four mixed-breed, medium-sized, 8-month-old dogs that tested negative for the presence of CCoV RNA in faeces and for CCoV antibodies in serum samples, were selected and randomly assigned to two groups of two dogs each. Dogs were housed individually in separate boxes, fed twice daily with a commercial dry dog food and provided water *ad libitum*. After an acclimatization period of 5 days, each dog was administered oronasally with 4 ml of a viral suspension of a CCoV field strain, with a titre of $10^{5.50}$ $TCID_{50}$ and 6.00×10^6 RNA copies/ml. To evaluate CCoV shedding, faecal samples were collected daily for 12 days from treated and non-treated CCoV-infected dogs and subjected to real-time RT-PCR analysis, as described below. For all experiments, humane standards were adhered to.

DNA and RNA synthesis

Cells were labelled for 24 h with ^{3}H-thymidine or ^{3}H-uridine (10 μCi/10^5 cells) and the radioactivity incorporated into acid-soluble and -insoluble material was determined as previously described [13]. Briefly, cells were washed three times with PBS and 0.4 ml 5% trichloroacetic acid (TCA) was added to each culture. The radioactivity in acid-soluble material was determined after 1 h at 4°C. Acid-insoluble radioactivity was measured after washing the TCA precipitates three times with ethanol, drying under an infrared lamp and dissolving the samples in 0.4 ml of a solution containing 0.1 M NaOH and 0.5% SDS.

Protein synthesis and PAGE analysis

CCoV- and mock-infected A72 cell monolayers were labelled with ^{35}S-methionine (10 μCi/10^5 cells, 2 h pulse) at 22 h post-infection (p.i.). At 24 h p.i., cells were lysed in L buffer (20 mM Tris-Cl pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1% NP40, 0.5% SDS), and the radioactivity incorporated was determined as described [11]. Samples containing the same amount of radioactivity were separated by SDS/PAGE (3% stacking gel, 10% resolving gel) and processed for autoradiography [11].

RNA extraction, Northern blot and real-time RT-PCR analysis

Total RNA from uninfected and virus-infected cells was isolated by the guanidinium isothiocianate method [13] and stored at –20°C. For detection of CCoV mRNA by Northern blot analysis, total RNA was fractionated (5 μg) on 1% agarose/formaldehyde gels and transferred

onto Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). For detection of CCoV mRNA, filters were hybridized with ^{32}P-labelled pCR21-M-CCoV probe [14]. After being stripped, filters were rehybridized with a plasmid specific for the β-actin gene 5′ end-labelled by T4 kinase with γ-AT^{32}P (Amersham), as a loading control [11]. Measurement of SARS-CoV genomic RNA was performed by quantitative real time RT-PCR using the commercial kit RealArt™ HPA-Coronavirus LC RT-PCR (Artus, Hamburg, Germany) on a LightCycler Instrument (Roche Diagnostic, Basel, Switzerland) [15]. For *in vivo* experiments, total RNA was extracted from each faecal sample with QIAamp® RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. The starting material consisted of 10 mg of faeces for each sample. Template RNAs were eluted in 50 µl of RNase-free water and stored at –70°C. Real-time RT-PCR analysis was performed as previously described [14].

Statistical analysis

Statistical analysis was performed using Student's *t*-test for unpaired data. Data were expressed as the mean ± SD and *P*-values of <0.05 were considered significant.

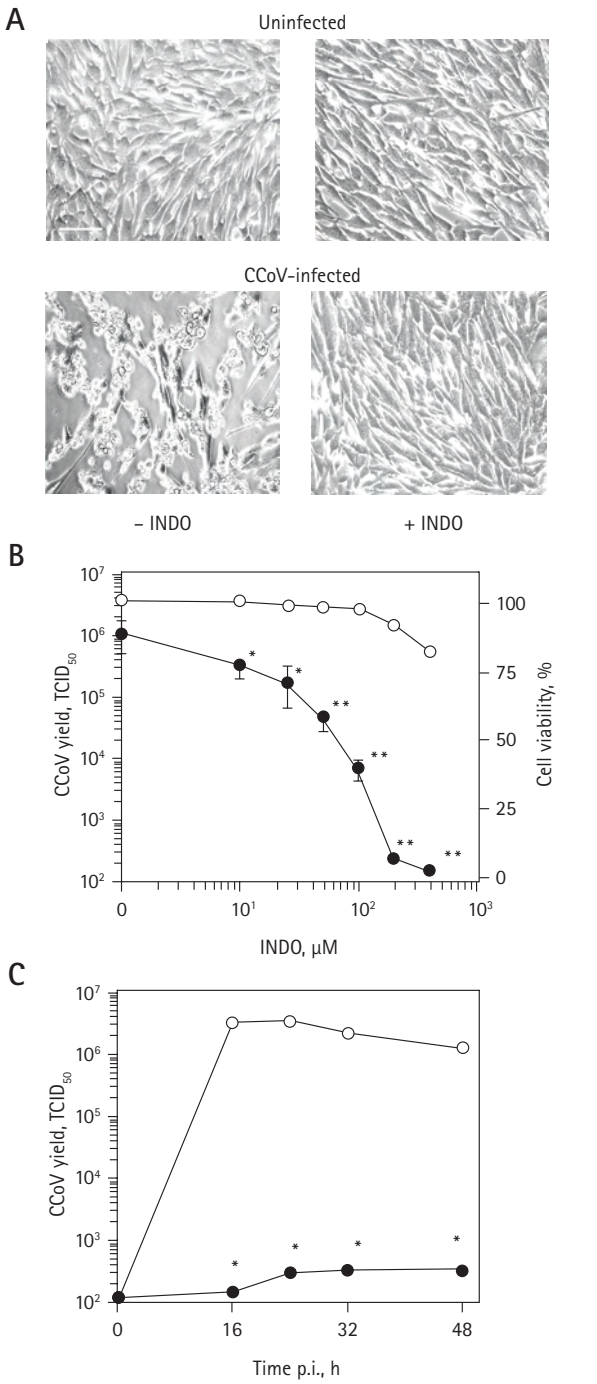
Results

INDO is a potent inhibitor of CCoV replication *in vitro*

Canine A72 cell monolayers were infected with CCoV (10 $TCID_{50}$/cell) and treated with different concentrations (10, 25, 50, 100, 200 and 400 µM) of INDO after the 1 h adsorption period. The effect of INDO on cell viability was determined by MTT assay in confluent cells. CCoV infection was highly cytopathic, causing cell shrinkage and loss of adhesion at 24 h p.i. (Figure 1A). Surprisingly, INDO was found to possess a remarkable antiviral activity, reducing viral particle production dose-dependently with an IC_{50} of 5 µM, an LD_{50} of 550 µM and a selectivity index of 110 (Figure 1B). At 400 µM, INDO caused a dramatic (more than 3 log) reduction in viral yield that lasted for at least 48 h p.i. (Figure 1C). At this concentration, INDO also had a remarkable cytoprotective effect in CCoV-infected cells, which did not show any sign of infection up to 36 h p.i. (Figure 1A). Treatment with RBV up to a concentration of 1 mM did not affect CCoV replication under the same conditions (data not shown). In addition, no effect on coronavirus replication was found in cells treated with the non-steroidal anti-inflammatory drug (NSAID) aspirin up to a concentration of 2 mM (Figure 2A), suggesting that INDO antiviral activity is independent of the block of cyclooxygenase function.

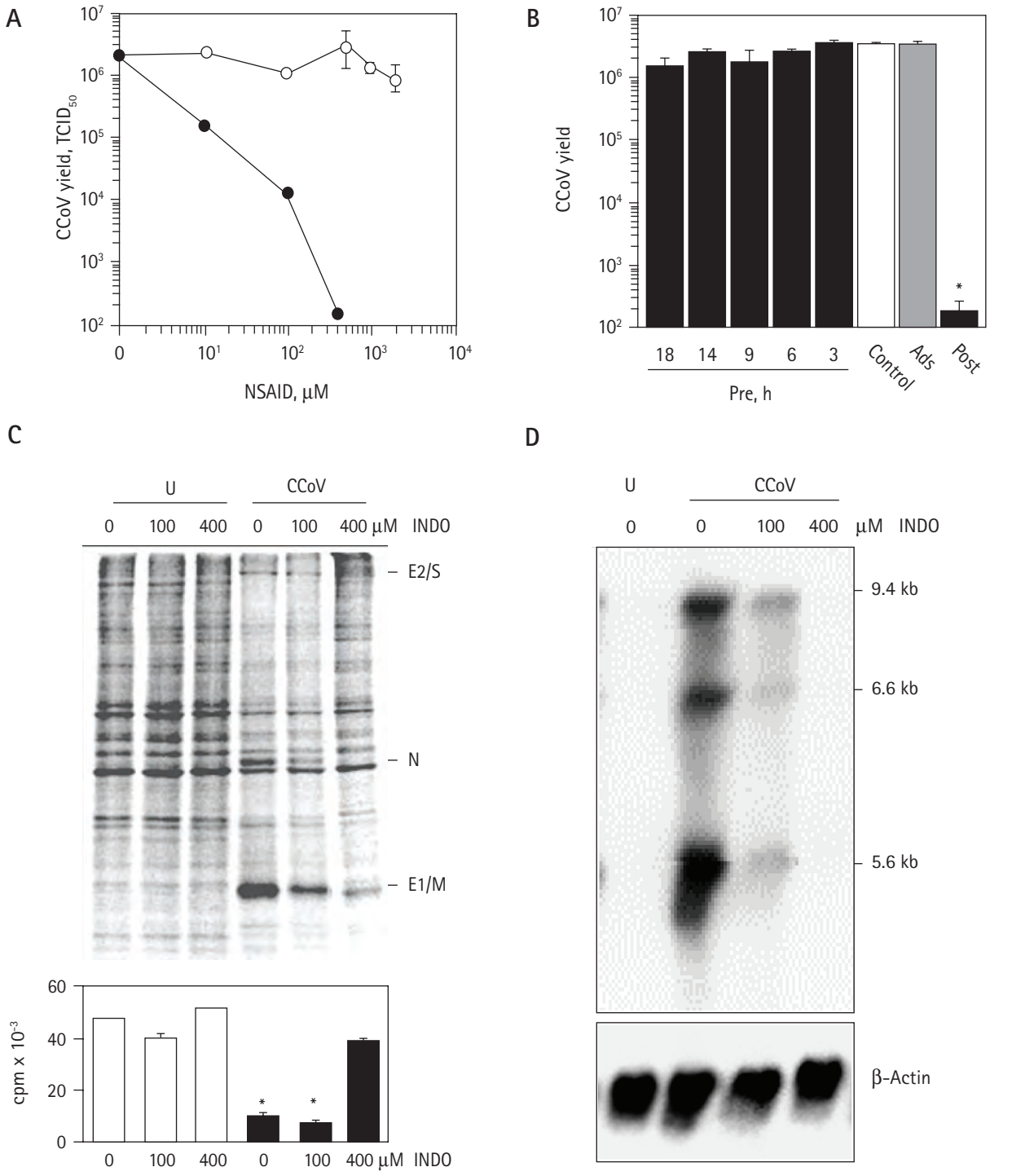
To investigate whether treatment of cells with INDO before virus adsorption was able to protect the cells

Figure 1. INDO is a potent inhibitor of CCoV replication



(A) Cytoprotective effect of INDO (400 µM) in CCoV-infected A72 cells 36 h p.i., bar, 50 µm. **(B)** CCoV yield (●) in the supernatant of infected cells treated with different concentrations (10, 25, 50, 100, 200 and 400 µM) of INDO, as determined by infectivity assay at 24 h p.i.. Data expressed in $TCID_{50}$/ml represent the mean ± SD of duplicate samples from a representative experiment of three with similar results. **P*=0.021; ***P*<0.001. Cell viability (○) determined by MTT assay in uninfected INDO-treated cells is expressed as percentage of MTT conversion in untreated control. **(C)** CCoV yield in the supernatant of infected cells treated with 400 µM INDO (●) or control diluent (○) was determined by infectivity assay at different times p.i.. Data expressed in $TCID_{50}$/ml represent the mean ± SD of three independent experiments. **P*<0.001. CCoV, canine coronavirus; INDO, indomethacin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; p.i., post-infection; TCID, tissue culture infective dose.

Figure 2. Inhibition of CCoV RNA and protein expression by indomethacin



(A) A72 cells were infected with CCoV and treated with different concentrations of the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin (●) (10, 100 and 400 μM) or aspirin (○) (10, 100, 400, 800 and 2,000 μM) after the 1 h adsorption period. CCoV yield was determined by infectivity assay in the supernatant of infected cells at 24 h p.i.. Data expressed in $TCID_{50}$/ml represent the mean ± SD of duplicate samples from a representative experiment of two with similar results. **(B)** A72 cells were treated with 400 μM INDO (filled bars) at the indicated times before infection (Pre), immediately after the adsorption period (Post) or only during the adsorption period (Ads, grey bar). The empty bar represents the untreated infected control (Control). CCoV yield was determined at 24 h p.i.. Data represent the mean ± SD of triplicate samples. **P*<0.001. **(C)** Uninfected (U, empty bars) or CCoV-infected (CCoV, filled bars) A72 cells treated with the indicated amounts of INDO were labelled with ^{35}S-methionine (2 h pulse) at 22 h p.i.. ^{35}S-Methionine incorporation into proteins is expressed as means ± SD cpm/10^6 cells (lower panel). **P*<0.001 as compared with uninfected control. Samples containing an equal amount of radioactivity were processed for SDS-PAGE analysis and autoradiography (upper panel). The major viral proteins E2/S, N and E1/M are indicated. **(D)** RNA extracted from uninfected (U) or CCoV-infected (CCoV) A72 cells, treated with the indicated amounts of INDO for 18 h, was analysed by Northern blot using a ^{32}P-labelled pCR21-M-CCoV probe (upper panel). Levels of β-actin mRNA in the same samples are shown as control (lower panel).

from viral infection, A72 cells were treated with 400 μM INDO for 18, 14, 9, 6 and 3 h. At the end of the treatment period, the drug was removed by washing the cell monolayers three times with culture medium, and cells were then infected with CCoV (10 $TCID_{50}$/cell). One set of cultures was treated with 400 μM INDO immediately after the virus adsorption period, as a positive control. As shown in Figure 2B, treatment of A72 cells with 400 μM INDO prior to viral infection had no effect on CCoV replication, indicating that, differently from interferon, INDO is not priming the host cell to raise a defence response against the invading virus. Moreover, treatment of the viral suspension with high concentrations (1 mM for 1 h) of INDO (data not shown) or the addition of 400 μM INDO only during the 1 h adsorption period had no effect on virus replication (Figure 2B), indicating that the drug is not affecting virus infectivity, binding or entry into target cells.

In the same experiment, INDO caused a dramatic (approximately four logs) reduction of virus yield when added soon after the adsorption period and kept for the next 24 h (Figure 2B). To investigate how early after infection INDO treatment needs to be started to be effective, CCoV-infected cells were treated with 400 μM INDO immediately after the adsorption period (time 0) or at 3, 6 and 12 h p.i., and virus yield were determined after 24 h. Treatment with INDO started at 0 or 3 h p.i. was extremely effective in inhibiting virus replication (control = $3.14 \pm 0.58 \times 10^6$ $TCID_{50}$/cell; INDO at time 0 = $<10^2$ $TCID_{50}$/cell; INDO at 3 h p.i. = $2.04 \pm 0.01 \times 10^2$ $TCID_{50}$/cell), further indicating that INDO is not affecting virus entry, and suggesting that the drug has no effect on virus uncoating. Treatment started at 6 h p.i. was less effective, but still able to inhibit virus replication (INDO at 6 h p.i. = $3.25 \pm 0.30 \times 10^3$ $TCID_{50}$/cell), whereas the drug lost most of its effect when administered at 12 h p.i. (INDO at 12 h p.i. = $2.15 \pm 0.80 \times 10^5$ $TCID_{50}$/cell). In a different experiment, to investigate whether the antiviral effect was retained upon removal of the drug, CCoV-infected cells were treated with 400 μM INDO soon after the adsorption period. At 3, 6 or 9 h p.i., the drug was removed by washing as described above, and culture medium devoid of the drug was added. Virus titres were determined at 24 h p.i.. Removal of the drug up to 9 h p.i. resulted in a complete reversal of the antiviral effect; treatments longer than 9 h were necessary in order to block virus replication (data not shown).

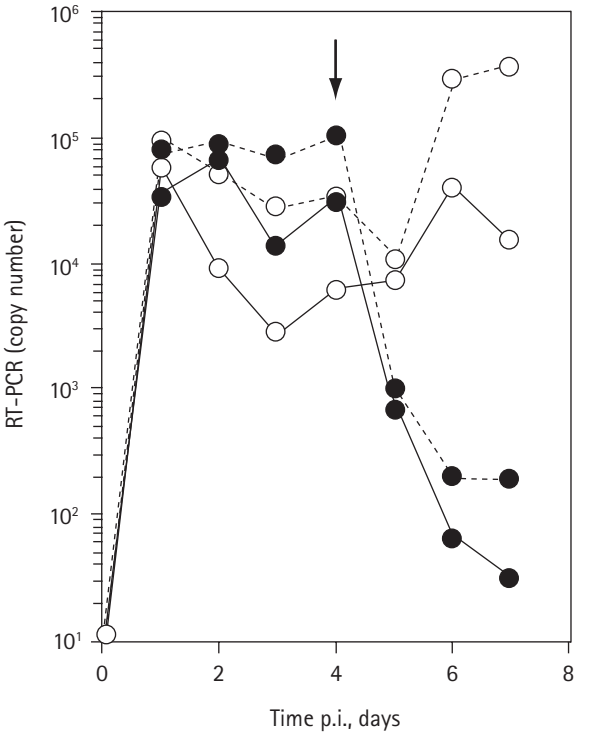
In order to determine whether INDO was affecting CCoV protein synthesis, confluent monolayers of A72 cells were mock-infected or infected with CCoV (10 $TCID_{50}$/cell) and treated with 100 or 400 μM INDO after the adsorption period. Uninfected and CCoV-infected cells were labelled with ^{35}S-methionine (2 h pulse) at 22 h p.i., and labelled proteins were analysed by SDS-PAGE and autoradiography. At this time, INDO did not inhibit protein synthesis in uninfected cells up to a concentration of 400 μM (Figure 2C, lower panel) and did not cause any detectable change in the pattern of cell protein synthesis (Figure 2C, upper panel). As expected, CCoV infection caused a dramatic inhibition of host cell protein synthesis (Figure 2C). In infected cells, INDO caused a dose-dependent inhibition of viral protein synthesis (Figure 2C, upper panel). CCoV proteins were barely detectable at concentration of 400 μM. At this concentration, INDO was also able to protect the host cell from the virus-induced shut-off of host protein synthesis (Figure 2C, lower panel).

CCoV RNA levels were then analysed in parallel samples by Northern blot analysis. Data shown in Figure 2D demonstrate that INDO profoundly affects viral RNA synthesis. No viral RNA is detected at the cytoprotective concentration of 400 μM, indicating that the drug-induced inhibition of virus protein synthesis is due to a dramatic decrease in the viral RNA levels.

Antiviral activity of INDO *in vivo*

In order to evaluate whether INDO could also be effective against coronaviruses *in vivo*, a group of six mixed-breed, medium-sized, 8-month-old dogs were tested for the presence of CCoV RNA in faeces by a real-time RT-PCR assay [14] and for CCoV antibodies in serum samples by an ELISA test [16]. Of these, four dogs that had tested negative for CCoV RNA and antibodies were selected and randomly divided into two groups (A and B). All animals were infected with CCoV ($10^{5.50}$$TCID_{50}$/dog) and dogs in group A were treated orally with INDO (1 mg/kg body weight) daily for 4 days, starting on day 4 p.i., whereas dogs in group B served as infected non-treated controls. The antiviral efficacy of INDO was determined by evaluating the CCoV shedding in the faeces of the infected dogs for a period of 12 days. In the first 2–3 days p.i., all infected dogs experienced mild diarrhoea, according to previous observations [14,17]. As shown in Figure 3, virus shedding in faeces was already detectable 1 day p.i., and the pattern was similar in all animals before starting treatment, with viral loads ranging from 10^4 to 10^5 RNA copies/μl, according to previous results [14]. Subsequently, the titre of shed virus in control non-treated dogs increased, reaching a peak at day 7 p.i. (mean titre = 2.11×10^5 RNA copies/μl). In contrast, in INDO-treated dogs, viral RNA titres in the faeces decreased rapidly after starting treatment, reaching minimal levels at day 7 p.i. (mean titre = 1.16×10^2 RNA copies/μl), in concomitance with the peak observed in non-treated dogs (Figure 3, closed circles). INDO antiviral effect was reversed upon suspension of treat-

Figure 3. Antiviral activity of INDO *in vivo*



Shedding of CCoV RNA in the faeces of dogs infected with CCoV and treated (●, group A) or non-treated (○, group B) with INDO (1 mg/kg body weight, oral administration) for 4 days. Arrow indicates the first day of treatment. Viral titres are expressed as RNA copy numbers per μl of template. Each sample was tested in duplicate. INDO, indomethacin.

ment. In fact, at the end of the 4-day treatment, CCoV RNA titres increased progressively, reaching the same values as control dogs on day 10 p.i. (data not shown). During and after the INDO treatment, no systemic or local adverse reactions were observed in treated dogs. These results indicated that INDO treatment is effective against coronavirus infection *in vivo* as well as *in vitro*.

INDO has a potent antiviral activity against SARS-CoV

On the basis of these observations, we investigated whether INDO could also inhibit SARS-CoV replication. VERO cell monolayers were infected with 2 $TCID_{50}$/cell SARS-CoV (TOR-2 isolate) for 30 min at 37°C, and then incubated in complete medium, containing different concentrations (100, 200 and 400 μM) of INDO or control diluent. In parallel, the effect of INDO (50, 100, 200 and 400 μM) on VERO cell viability was determined by MTT assay on mock-infected VERO cell monolayers. As shown by infectious virus titration, INDO inhibited SARS-CoV replication dose-dependently with an IC_{50} of 50 μM (Figure 4A, closed circles). A greater than 99% decrease in virus yield was detected at concentrations that were non-toxic for uninfected cells as shown by MTT assay (Figure 4A, open circles). INDO treatment also partially protected cells by the cytopathic effect caused by SARS-CoV infection for a period of 24 h (Figure 4B). To determine whether treatment with INDO could inhibit cellular DNA or protein synthesis, in a parallel experiment VERO cells were treated with different concentrations (50, 100, 200 and 400 μM) of INDO and then labelled with ^{3}H-thymidine (10 μCi/10^5 cells) or ^{35}S-methionine (10 μCi/10^5 cells) for the next 24 h. At this time, the radioactivity incorporated into acid-insoluble material was determined. As shown in Figure 4C, INDO did not significantly alter DNA or protein synthesis in uninfected cells up to a concentration of 400 μM.

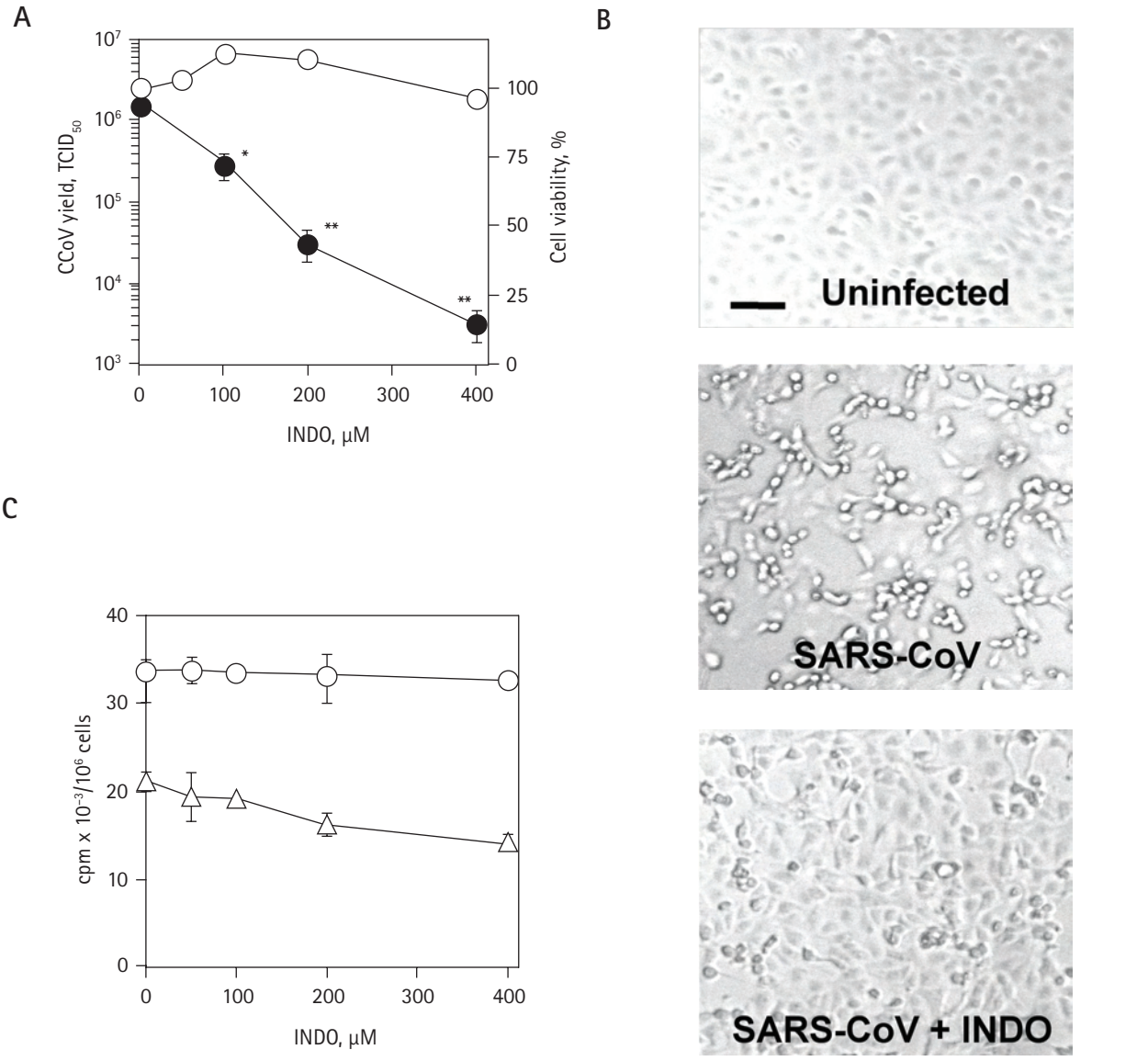
Since we have recently set up a model of SARS-CoV infection in human lung epithelial cells (A549 cell line), we investigated whether INDO was also able to inhibit SARS-CoV replication in human cells. The results indicated that INDO is able to inhibit SARS-CoV replication at the same concentrations as in VERO cells (control cells = $4.39 \pm 1.2 \times 10^3$; 100 μM INDO treated cells = $<10^2$ $TCID_{50}$/ml), demonstrating that the antiviral activity of INDO is not dependent on the type of cells.

Next, we investigated the effect of IFN and RBV, which have been used in the clinic to treat SARS-CoV infection [18], in the VERO cell model. VERO cell monolayers were infected with 2 $TCID_{50}$/cell SARS-CoV for 30 min at 37°C, and then incubated in complete medium containing different concentrations of IFN-α (500 and 5,000 IU/ml), RBV (40 and 400 μM) or INDO (100 and 400 μM). The results shown in Figure 5A indicate that treatment with IFN-α or RBV after the adsorption period had no major effect on SARS-CoV replication up to a concentration of 5,000 IU/ml for IFN-α and 400 μM for RBV, respectively. The effect of the NSAID aspirin was then investigated in a parallel experiment. As shown above for the canine coronavirus (Figure 2A), treatment with aspirin had no effect on SARS-CoV replication up to a concentration of 2 mM (Figure 5B).

Treatment of SARS-CoV suspension for 1 h with 1 mM INDO (data not shown), or the addition of 300 μM INDO prior to viral infection and/or during the 1 h virus adsorption period had no effect on virus yield, indicating that, as shown above for CCoV, INDO is not affecting SARS-CoV binding or entry into target cells (Figure 5C).

As for other coronaviruses, the expression of the SARS-CoV genome is mediated by translation of the genomic RNA and a 'nested' set of subgenomic messenger RNAs, produced by a unique mechanism involving discontinuous transcription during negative-

Figure 4. Antiviral activity of INDO during SARS-CoV infection



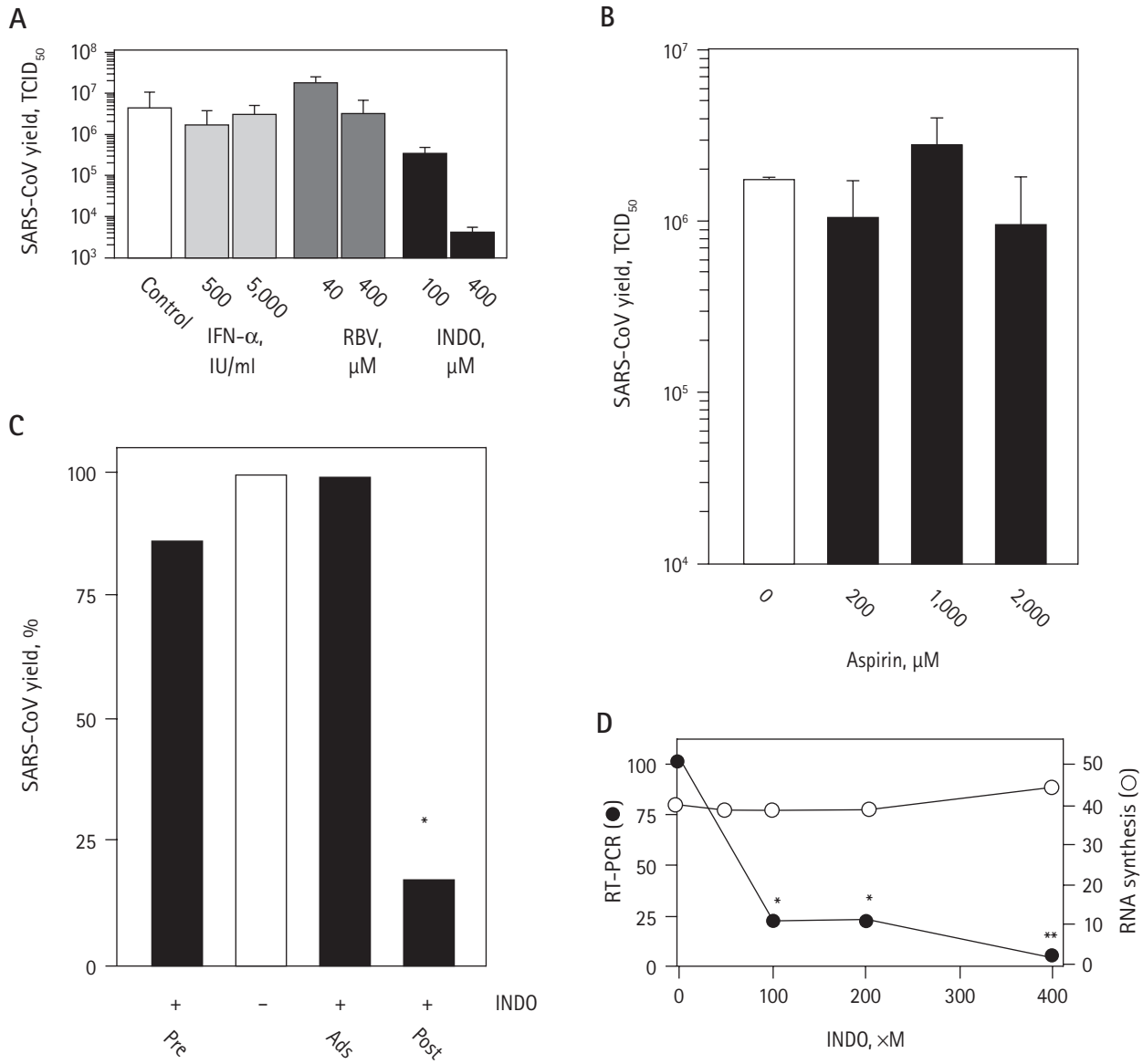
VERO cells infected with SARS-CoV (2 $TCID_{50}$/cell) were incubated in complete MEM medium containing the indicated amounts of INDO or control diluent for 48 h. **(A)** SARS-CoV titres (●) in $TCID_{50}$/ml represent the mean ± SD of duplicate samples from a representative experiment of three with similar results. **P*<0.003; ***P*<0.0001. Cell viability (○) determined by MTT assay in uninfected cells is expressed as in Figure 1. **(B)** Cytoprotective effect of INDO (400 μM) in SARS-CoV-infected VERO cells at 24 h p.i., bar, 100 μm. **(C)** Effect of INDO on DNA and protein synthesis in uninfected cells as determined by ^{3}H-thymidine (○) and ^{35}S-methionine (△) incorporation (24 h pulse), and expressed as mean ± SD cpm/10^{6} cells. INDO, indomethacin.

strand RNA synthesis [3]. To determine whether, as in the case of CCoV, INDO was acting by blocking viral RNA synthesis, VERO cells were infected with SARS-CoV and treated with different concentrations (100, 200 and 400 μM) of INDO soon after the virus adsorption period. Total RNA was extracted 24 h p.i. and analysed by RT-PCR. In order to investigate whether INDO was affecting cellular RNA synthesis, mock-infected cells were treated identically in parallel and labelled with ^{3}H-uridine (10 μCi/10^{5} cell) for the next 24 h. As shown above for CCoV, INDO treatment caused a decrease of intracellular SARS-CoV RNA levels dose-dependently, reaching an inhibition of more than 95% of control at concentrations of INDO that did not affect RNA synthesis in uninfected cells (Figure 5D).

Discussion

Coronaviruses cause a wide spectrum of diseases in humans and animals, primarily infecting the respira-

Figure 5. INDO inhibits SARS-CoV RNA expression



(A,B) SARS-CoV-infected VERO cells were treated with different concentrations of IFN-α, RBV, INDO (A) or aspirin (B) soon after the adsorption period. SARS-CoV yield was determined at 24 h p.i.. Data expressed as $TCID_{50}$/ml represent the mean ± SD of duplicate samples from a representative experiment. The empty bar represents the untreated infected control. **(C)** VERO cells were treated with 300 μM INDO (filled bars) 9 h before virus infection (Pre), only during the adsorption period (Ads) or soon after virus adsorption (Post). The empty bar represents the untreated infected control (–). SARS-CoV yield was determined at 36 h p.i.. Data are expressed as percent of virus yield in untreated cells. **P* =0.0002. Untreated infected control = 1.74×10^6 $TCID_{50}$/ml. **(D)** Intracellular SARS-CoV replication as measured by real time RT-PCR targeted to the viral polymerase gene and expressed as percentage of genomic RNA copy number in untreated infected cells (●). **P*<0.025; ***P*=0.017. Untreated infected control = 1.696×10^3 copies/cell. RNA synthesis in uninfected cells was assayed by ^{3}H-uridine labelling (24 h pulse). Data are expressed as ^{3}H-uridine incorporation/total cellular uptake ratio (○). IFN-α, interferon α; INDO, indomethacin; RBV, ribavirin.

tory and gastrointestinal mucosa; however, it was not until the SARS epidemic that a large scientific interest focused on this family of viruses. The human coronaviruses usually cause mild self-resolving upper respiratory tract infections, most of which result in the common cold, and have occasionally been associated with pneumonia or with neurological symptoms and myocarditis [3]. Before the SARS epidemic, no effective specific antiviral therapy for coronavirus infections was known.

The results described in the present report demonstrate that the NSAID INDO has a potent antiviral activity against different coronaviruses, being effective against the canine (CCoV) and the human (SARS-CoV) coronaviruses. A dramatic antiviral effect was also found in a model of *in vitro* feline coronavirus infec-

tion, FCoV type II in feline kidney CRFK cells (C Amici and MG Santoro, unpublished observations). In addition, oral administration of INDO at the concentration of 1 mg/kg body weight, which is in the range of the therapeutic dose in humans [19] and dogs [20], to CCoV-infected dogs was found to cause a dramatic decrease in virus titres in the faeces of the treated animals during a 4-day treatment period, also demonstrating a potent antiviral activity of the drug *in vivo*. No local or systemic adverse reactions were observed during or after treatment with INDO. It should be pointed out that, since coronavirus infection in dogs causes a mild disease characterized by the occurrence of moderate diarrhoea followed by a rapid recovery of the infected animals, this model does not allow us to define the effectiveness of INDO on the outcome of the disease, but only the effect of the drug on virus replication. In addition, due to the difficulty in finding pups negative for CCoV RNA and anti-CCoV antibodies (C Buonavoglia, personal communication), only a small number of animals could be tested.

The mechanism of antiviral action appears to be novel. We have shown that treatment of target cells with INDO prior to viral infection has no effect on coronavirus replication, indicating that, as opposed to IFN, INDO is not priming the host cell to raise a defence response against the invading virus. Moreover, INDO does not affect virus infectivity, binding or entry into target cells, but acts early on the coronavirus replication cycle, selectively blocking viral RNA synthesis.

INDO has been used for a long time as a potent anti-inflammatory drug, acting by blocking COX-1 and COX-2 activity and inhibiting pro-inflammatory prostaglandin synthesis [21]. The antiviral effect, however, appears to be cyclooxygenase-independent, since it occurs at concentrations higher than those needed for COX inhibition (10^{-8},10^{-9}M) [22]; in addition, the antiviral activity cannot be mimicked by the potent COX inhibitor aspirin, which has no effect on either CCoV or SARS-CoV replication up to millimolar concentrations.

Although the mechanisms underlying the antiviral activity of INDO against coronavirus infection require further investigation, the results shown herein may have important implications. In fact, SARS resurgence is still a threat, since no definitive animal reservoir has been identified, and sporadic cases have been reported since the epidemic period [4,5]. The recently reported evasion of antibody neutralization by SARS-CoV raises concerns about the efficacy of SARS-CoV vaccines [23]. Moreover, although different clinical approaches to SARS therapy have been tried using steroidal anti-inflammatory agents and broad-spectrum antivirals such as RBV and IFN, no effective treatment protocol has been established [18]. As reported above, surprisingly, INDO was found to be a more effective antiviral agent than IFN-α up to a concentration of 5,000 IU/ml [23], and RBV up to concentrations of 400 μM. In addition INDO was also found to be effective during SARS-CoV infection of human pulmonary epithelial cells.

Altogether, the results described suggest that indomethacin, possessing both anti-inflammatory properties and a direct antiviral activity against SARS-CoV, could be effective in the treatment of SARS.

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