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Antiproliferative effects of carbon monoxide on pancreatic cancer

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ABSTRACT

Background: Carbon monoxide, the gaseous product of heme oxygenase, is a signalling molecule with a broad spectrum of biological activities. The aim of this study was to investigate the effects of carbon monoxide on proliferation of human pancreatic cancer.
Methods: In vitro studies were performed on human pancreatic cancer cells (CAPAN-2, BxPc3, and PaTu-8902) treated with a carbon monoxide-releasing molecule or its inactive counterpart, or exposed to carbon monoxide gas (500 ppm/24 h). For *in vivo* studies, pancreatic cancer cells (CAPAN-2/PaTu-8902) were xenotransplanted subcutaneously into athymic mice, subsequently treated with carbon monoxide-releasing molecule (35 mg/kg b.w. i.p./day), or exposed to safe doses of carbon monoxide (500 ppm 1 h/day; $n = 6$ in each group).
Results: Both carbon monoxide-releasing molecule and carbon monoxide exposure significantly inhibited proliferation of human pancreatic cancer cells ($p < 0.05$). A substantial decrease in Akt phosphorylation was observed in carbon monoxide-releasing molecule compared with inactive carbon monoxide-releasing molecule treated cancer cells (by 30–50%, $p < 0.05$). Simultaneously, carbon monoxide-releasing molecule and carbon monoxide exposure inhibited tumour proliferation and microvascular density of xenotransplanted tumours ($p < 0.01$), and doubled the survival rates ($p < 0.005$). Exposure of mice to carbon monoxide led to an almost 3-fold increase in carbon monoxide content in tumour tissues ($p = 0.006$).
Conclusion: These data suggest a new biological function for carbon monoxide in carcinogenesis, and point to the potential chemotherapeutic/chemoadjuvant use of carbon monoxide in pancreatic cancer.

1. Introduction

For decades, carbon monoxide (CO) exposure has been considered a potential threat to human health, and the endogenous production of this gaseous molecule was only thought to be a waste product, a biologically inactive by-product of heme catabolism. However, it is now widely accepted that CO formed endogenously by heme oxygenase (HMOX) confers cytoprotection against tissue and cellular injury [1,2]. CO acts as a smooth muscle relaxant as well as an inhibitor of platelet aggregation *via* guanylate cyclase and cGMP generation [2]. The functional properties of CO have often been compared with nitric oxide (NO), another endogenous gaseous molecule. Indeed, CO shares a number of biological functions analogous to NO [1]. The ability of both NO and CO to act as a vasodilator and to modulate endothelial cell permeability makes it plausible that they could also play essential roles in angiogenesis. It has previously been reported that NO has a dual effect on angiogenesis, and can either promote or inhibit angiogenesis in a dose-dependent manner [3]. Although certain studies have reported that CO is pro-angiogenic [4], the similarity of CO to NO could lead to the speculation that CO might also inhibit carcinogenesis, at least partially, *via* the suppression of angiogenesis. Under stress conditions, the production of CO is increased owing to the upregulation of the stress-responsive heme oxygenase isoenzyme,

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HMOX1 (OMIM*141250) [5]. The recent discovery of transition metal carbonyls that act as CO-releasing molecules (CORMs) has provided a new impetus for the investigation of CO as a cellular messenger, as well as a potential therapeutic agent [6,7]. Both CORMs and CO at doses free from toxic side-effects have been shown to exert important biological functions in numerous model *in vitro* and *in vivo* systems, including vasodilating, antiproliferative, anti-inflammatory effects, contributing to the amelioration of many pathological conditions such as ischaemia – reperfusion injury, inflammatory bowel disease, and organ rejection (for a comprehensive review of the biological effects of CO, see Motterlini and Otterbein [7]). A wide range of CORMs/CO dosages have been tested in these models, depending on CORM type, the means of delivery, and the model used [7]. Significantly, the CO delivered was demonstrated to be non-toxic for healthy tissues, when keeping the CO haemoglobin levels within safe levels [8]. Based on these data, a CO inhalation system for human use has been developed and used in the first clinical trials [7].

Pancreatic tumours, having high mortality and recurrence rates, are an example of a tumour type in which any type of medical therapy has, at best, been only modestly effective [9]. Thus, the effective therapy for pancreatic cancer depends on the search for alternative therapeutic modalities that have the potential to inhibit multiple signalling pathways. Although pancreatic carcinogenesis is a very complex issue, with numerous intracellular pathways involved, the phosphatidylinositol-3 kinase/Akt (protein kinase B) seems to play a key role [10]. Akt activation is frequent in pancreatic cancer and correlates well with prognosis [11]; its inhibition has been reported to sensitise cancer cells to the tumour-suppressive effects of chemotherapy [12,13].

All of these facts led us to investigate the potential antiproliferative effects of CO and/or CORM-2 (a ruthenium-based, lipid-soluble CORM) on human pancreatic cancer, using experimental *in vitro* and *in vivo* models, with a special focus on the possible CO-mediated effects on Akt phosphorylation.

2. Methods

2.1. Reagents

All cell culture reagents and chemicals, and tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (more commonly known as CORM-2), were obtained from Sigma Aldrich (Prague, Czech Republic). The CO (500 ppm) gas mixture (20% O_2, 0.03% CO_2, remainder nitrogen) for *in vitro* studies was obtained from Linde Gas (Prague, Czech Republic).

2.2. Cell cultures

The pancreatic cancer cell lines CAPAN-2, BxPc3 (ATCC, Manassas, VA, USA), and PaTu-8902 (DSMZ, Braunschweig, Germany) were used for the *in vitro* studies. The cell lines were cultured as described previously [14]. The cell suspensions ($2 \times 10^{5-6}$ cells/ml) were used for the inoculation of individual wells in the 6-well plate. Cells were treated with either a ruthenium-based CORM (CORM-2, 50 μmol/L; this relatively high concentration was used for all *in vitro* studies because of the very short half-life of CORM-2 [7]) or its inactive counterpart, iCORM-2 (a CO-free CORM-2). The use of iCORM-2 as a control is important, since the Ru-based carrier molecule may exert some biological properties itself. Either the CORM-2 or iCORM-2 was dissolved in a solution of DMSO in PBS (final concentration of DMSO did not exceed 1%, vol/vol), on a daily basis for a period of three days. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 was prepared by leaving CORM-2 at room temperature for 2 days, and flushing with nitrogen to remove the residual CO [6]. Before initiating the experiments, the release of CO from freshly diluted CORM-2 was confirmed by gas chromatography (for methodology see below). Alternatively, pancreatic cancer cells, cultured in an analogous manner to that described above, were directly exposed to CO (500 ppm) for 24 h using specific air jars (Oxoid CZ, Thermo Fisher Scientific, Prague, Czech Republic). After treatment, the cells (experiments were performed in triplicate) were washed with PBS, harvested by 0.25% trypsin, and re-suspended. Both cell growth and viability were assessed by the direct counting of trypan blue dye (0.4%) excluding cells.

To study the distribution of CO within the cells exposed to CORM, CORM-2 was incubated with PaTu-8902 pancreatic cancer cells for 75 min at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 and 1% DMSO in PBS were used as controls. The CO concentration in the cells and media were measured as described below.

2.3. Determination of Akt phosphorylation

Akt phosphorylation in cancer cell lysates was determined by ELISA (based on anti-phospho-Akt Ser473 antibody, SuperArray Bioscience Corporation, MD, USA), after treatment of CAPAN-2 pancreatic cancer cells with CORM-2/iCORM-2 (50 mol/L) for 75 min, according to the manufacturer's instructions. Cells exposed to 1% DMSO in PBS (solvent for CORM-2) were also compared with untreated cells. Experiments were performed in hexaplets.

Simultaneously, Western blot analyses of phosphorylated Akt protein were performed on the CAPAN-2 and PaTu-8902 pancreatic cancer cells, treated in an identical manner (CORM-2/iCORM-2, 50 mol/L, 75 min incubation). The cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, MA, USA), according to the manufacturer's instructions. Protein concentration was determined using a BCA assay (Thermo Scientific, IL, USA). Thirty micrograms of protein lysate were separated by SDS-PAGE electrophoresis (10% gel). The proteins were transferred to a PVDF membrane and then immunoblotted with anti-phospho-Akt (Ser473), anti-Akt, and anti-β-actin (Cell Signaling Technology, MA, USA). Antibodies were detected using Goat Anti-Rabbit IgG H&L (HRP) antibody (Abcam, UK), and analysed by ECL (LumiGLO®, Cell Signaling Technology, MA, USA). A Fusion Fx7 device and Bio-1D software (Vilber Lourmat, France) were used to quantify the signals. Results were expressed as the percentage of the total Akt level that was phospho-Akt.

2.4. In vivo tumour models

Six- to eight-week-old athymic mice (strain CD-1, Charles River WIGA, Sulzfeld, Germany) were transplanted subcutaneously with either 10^7 human CAPAN-2 or PaTu-8902 pancreatic cancer cells mixed with matrigel. Seven to ten days after tumour cell implantation, the CAPAN-2-bearing mice received a daily intra-peritoneal treatment of either CORM-2 or iCORM-2 ($n = 6$ in each group) dissolved in 1% DMSO in PBS (35 mg/kg), whereas the PaTu-8902-bearing mice were exposed for 1 h daily to either 500 ppm CO in the synthetic air or ambient air *per se*. The primary outcome of this type of *in vivo* study was the survival time; tumour progression was assessed simultaneously as well (tumour size was monitored every three days in all groups, and the tumour volume determined as described previously [15]). In an additional *in vivo* study, the animals treated with CORM-2 or iCORM-2 ($n = 6$ in each group) were sacrificed at day 14 for the quantification of the capillary density of the tumour. Finally, other sets of animals ($n = 6$ for each group) were exposed to CO (500 ppm of CO in synthetic air for 1 h a day). These animals were either sacrificed immediately after the last CO exposure, for the determination of COHb and CO content in the tumour

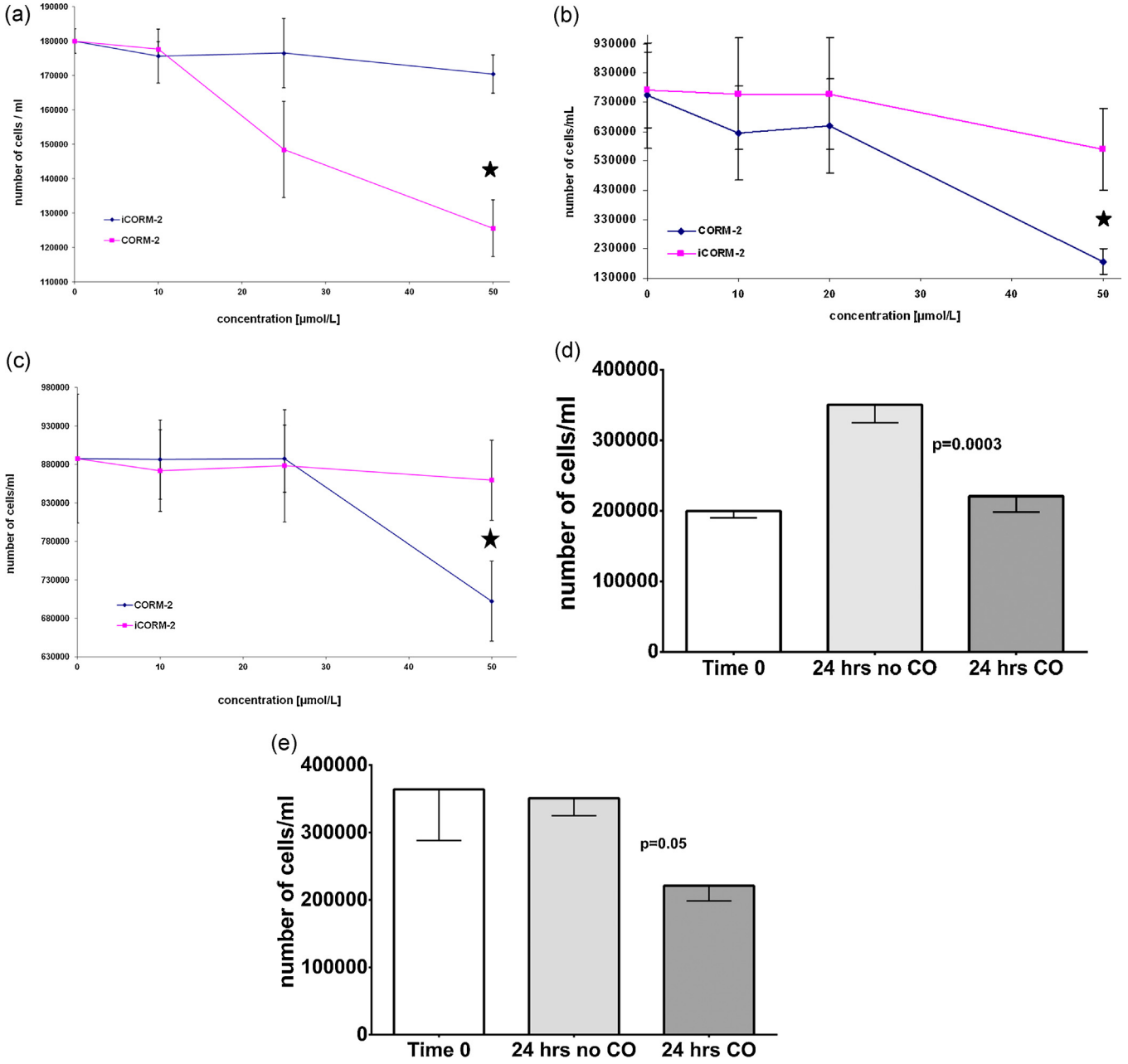

Fig. 1. CO inhibits pancreatic cancer cell proliferation *in vitro*. The effect of CORM-2 (48-h exposure) on pancreatic cancer cell lines: (a) PaTu-8902; (b) CAPAN-2; (c) BxPc3. $^{*}p < 0.05$ (CORM-2 treatment *vs.* iCORM-2, based on ANOVA on Ranks with Dunn's *post hoc* testing). ANOVA, analysis of variance; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM. The effect of 24-h CO exposure (500 ppm) on the proliferation of human pancreatic cancer cell lines: (d) PaTu-8902; (e) CAPAN-2; $n = 6$ for each group. CO, carbon monoxide.

tissue; or with an 8-h latency after the last exposure, to assess the long-term distribution of CO in various organs (liver, heart, spleen, kidneys, lung and brain).

The local animal research committee approved the protocols for all aspects of the animal studies in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health.

2.5. CO measurement

For the determination of CO in the tissues, samples (150–200 mg) were harvested, thoroughly washed in heparinized saline, diluted 1:4 (w/w) in ice-cold reaction buffer (0.1 M PBS, pH 7.4), diced, and sonicated with an ultrasonic tissue disruptor (model XL2000, Misonics, Framingdale, NY, USA). Forty microliters of tissue sonicate were added to CO-free, septum-sealed vials containing 5 μL of 30% (w/v) sulphosalicylic acid. After 30 min incubation on ice, the CO released into the vial headspace was quantified by gas chromatography with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA), as previously described [16]. This method has a detection limit of 1 pmol of CO, with a linear range of 1–500 pmol of CO. Tissue CO content was calculated as pmol of CO per mg of tissue. CO content in the cells (expressed as pmol of CO per mg of protein) and in the media from the *in vitro* experiments were measured in an analogous way.

To determine CO liberation from CORM-2, CORM-2 or its inactive counterpart (iCORM2) were dissolved in 1% DMSO in PBS and immediately mixed with the mouse blood in a CO-free vial. Next,

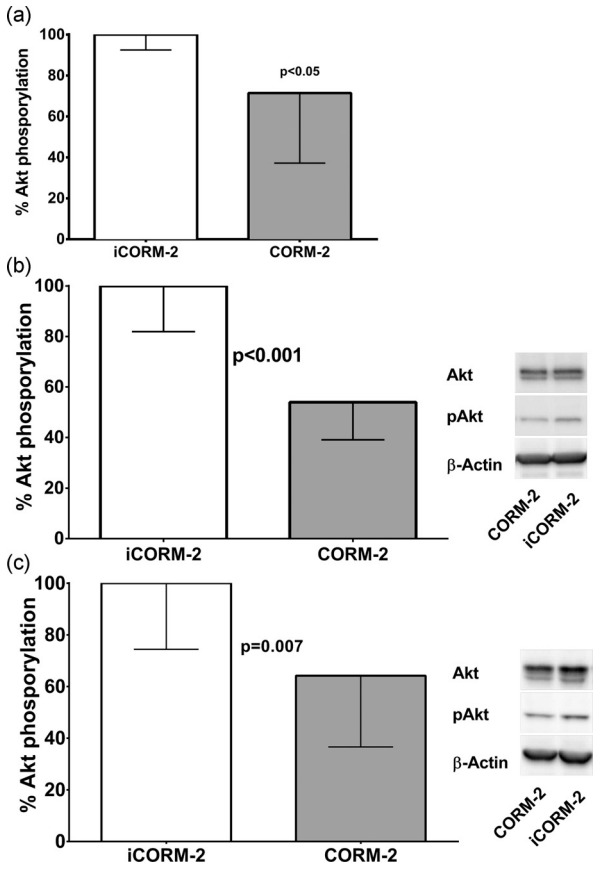

Fig. 2. CORM-2 inhibits Akt protein phosphorylation in pancreatic cancer cells. (a) Akt protein phosphorylation in CAPAN-2 cell lysates determined by ELISA. (b) Akt protein phosphorylation in CAPAN-2 cell lysates determined by Western blot. (c) Akt protein phosphorylation in PaTu-8902 cell lysates determined by Western blot. CAPAN-2 and PaTu-8902 pancreatic cancer cells were exposed to CORM-2 (50 μmol/L, 75 min exposure); data expressed as mean ± SD; $n = 6$ for each group. Two bands of Akt protein correspond to different Akt isoforms [36]. CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM.

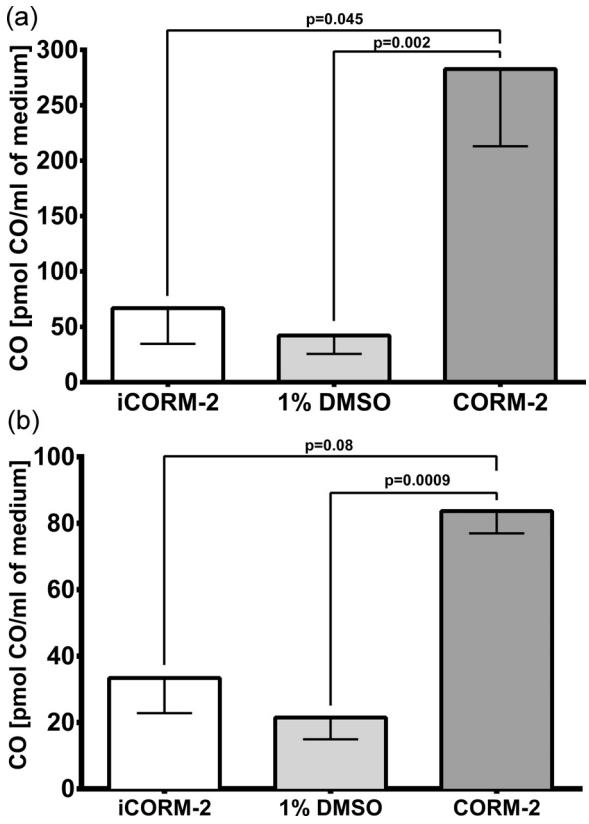

Fig. 3. CO distribution in the PaTu-8902 pancreatic cancer cells exposed to CORM-2. (a) CO content within PaTu-8902 pancreatic cancer cells exposed to CORM-2 (50 mol/L, 75-min exposure). (b) CO content in the culture media of PaTu-8902 pancreatic cancer cells exposed to CORM-2 (50 μmol/L, 75-min exposure); $n = 6$ for each group. CO, carbon monoxide; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM.

CO-haemoglobin formation was measured by gas chromatography as previously described [17]. The CO-haemoglobin method has a detection limit of 1 pmol of CO per vial.

2.6. Immunohistochemistry and quantification of capillary density

The immunohistochemistry was performed as previously described [18] on frozen or formalin-fixed, paraffin-embedded, randomly sampled, 3-μm sections of tumours with a 1:100 dilution of rat anti-mouse CD31 (BD Biosciences Oxford, UK), peroxidise-labelled rabbit anti-rat antibody (1:250). The mean density of CD31-positive vessels was determined in two areas per section per animal, with these images captured and analysed using Image Pro Plus image analysis software (Media Cybernetics Europe, Berkshire, UK).

2.7. Statistical analyses

All data are presented as the mean ± SD, or median and 25–75% range, when the data were non-normally distributed. Statistical comparisons of *in vitro* data were performed using ANOVA followed by the Student–Newman–Keuls test, or ANOVA on ranks with Dunn's *post hoc* testing, as appropriate. The statistical significance of differences between *in vivo* variables was evaluated by t-test or the Mann–Whitney rank sum test. Kaplan–Meier log rank survival analysis with Holm–Sidak *post hoc* testing was used to assess the effect of CORM-2 treatment on the survival of the animals. Group mean differences in tumour size were measured by repeated measures analysis of variance (RM ANOVA) with Holm–Sidak *post hoc* testing. When needed, log transform values of tumour size were used for comparisons to comply with equal variance requirements. Statistical significance was set at the value of $p < 0.05$.

3. Results

3.1. CO inhibits pancreatic cancer cell proliferation in vitro

Carbon monoxide, in the form of CORM-2, markedly inhibited proliferation of all tested human pancreatic cancer cell lines in a dose-dependent manner ($p < 0.05$, Fig. 1a–c).

To analyse whether CO in the form of CORM-2 might affect phosphorylation of the Akt protein (one of the key events in pancreatic carcinogenesis [10–13]), CAPAN-2 pancreatic cancer cells were treated with CORM-2 (50 μmol/L). Consistent with its antiproliferative effects, this concentration of CORM-2 was found to inhibit Akt phosphorylation in CAPAN-2 and PaTu-8902 pancreatic cancer cells by 30–50% compared with iCORM-2 (its inactive counterpart), as confirmed by both ELISA and Western blotting analyses ($p < 0.05$, Fig. 2a–c).

In order to assess whether the observed antiproliferative effects were only related to CO released from the CORM-2 molecule, PaTu-8902 and CAPAN-2 pancreatic cancer cells were directly exposed to CO in synthetic air (500 ppm) for 24 h. In these studies, CO clearly prevented proliferation of the PaTu-8902 cells ($p < 0.05$, Fig. 1d),

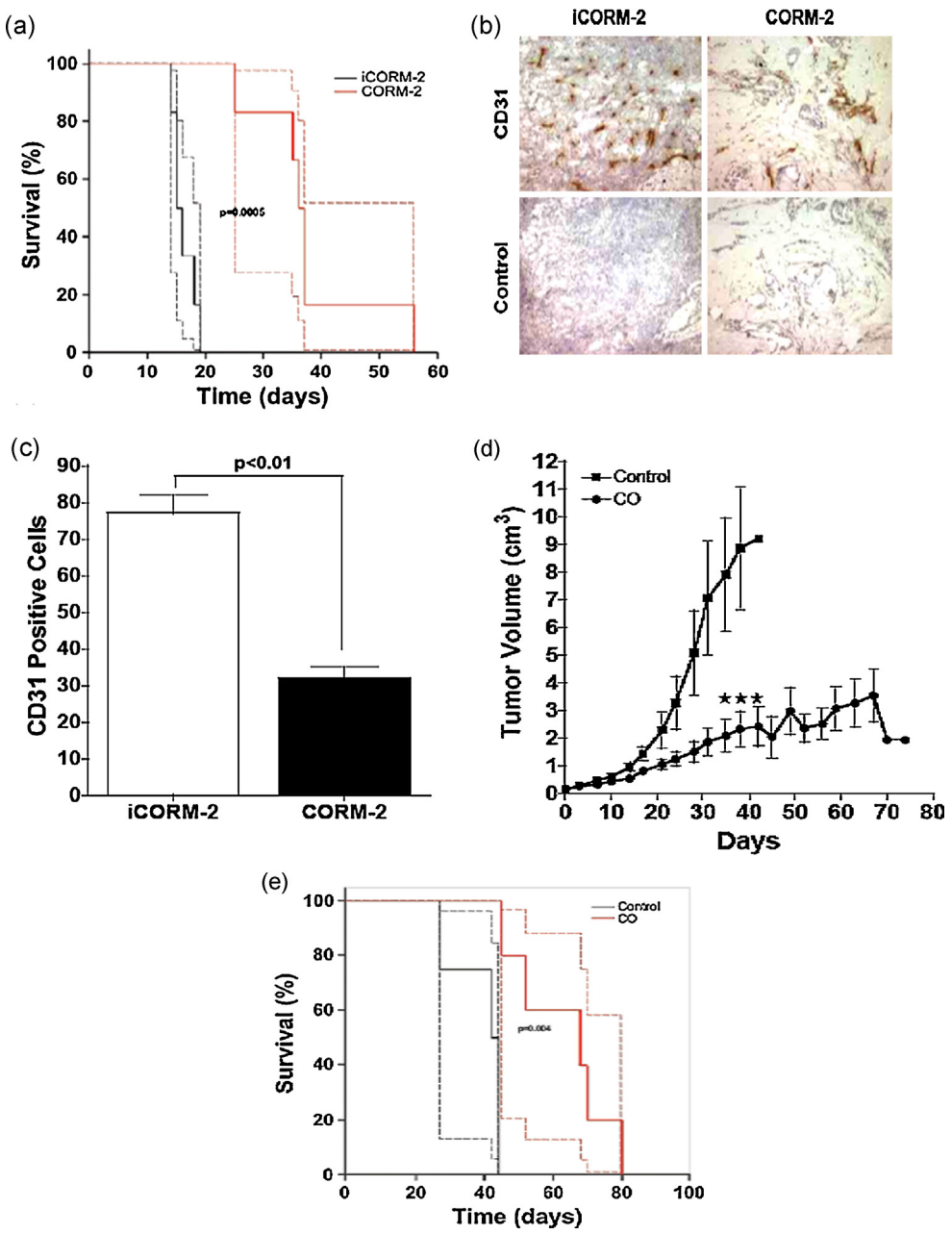

Fig. 4. CO inhibits pancreatic tumour progression *in vivo*. (a) Survival of athymic mice bearing CAPAN-2 human pancreatic cancer cells treated with CORM-2 (35 mg/kg/day, i.p. administration). Data expressed as mean ± SD, graphs represent survival times (continuous line) and respective upper and lower confidence intervals (dashed lines), $n = 6$ for each group. (b) Immunohistochemical localisation and (c) quantification of CD31-positive vessels in CAPAN-2 tumours from mice after 14 days of treatment with CORM-2. (d) Tumour size in athymic mice bearing PaTu-8902 human pancreatic cancer cells exposed to CO (500 ppm CO for 1 h/day). $^*p < 0.05$ (CO treatment *vs.* control, based on RM ANOVA with Holm–Sidak *post hoc* testing). (e) Survival curve of athymic mice bearing PaTu-8902 human pancreatic cancer cells exposed to CO (500 ppm CO for 1 h/day). Data expressed as mean ± SD, graphs represent survival times (continuous line) and respective upper and lower confidence intervals (dashed lines), $n = 6$ for each group. ANOVA, analysis of variance; CO, carbon monoxide; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM; SD, standard deviation; ppm, parts per million.

and was even able to induce apoptosis in the CAPAN-2 pancreatic cancer cell line ($p < 0.05$, Fig. 1e).

Analysis of CO in the PaTu-8902 pancreatic cancer cells, exposed to 50 μmol/L of CORM-2, revealed a substantial increase in CO concentration within the cells (Fig. 3a) as well as the culture media (Fig. 3b).

3.2. CO inhibits pancreatic tumour growth in vivo

To confirm our *in vitro* data, the anticancer effects of CO were validated in an experimental animal cancer model, represented by athymic mice xenografted subcutaneously with human pancreatic cancer cells (CAPAN-2/PaTu-8902).

In the first model, athymic mice carrying CAPAN-2 xenografts were administered a dose of 35 mg/kg CORM-2 intraperitoneally, on a daily basis. Treatment with CORM-2 induced a significant increase in the survival rate, compared with the iCORM-2 treated control animals (37.7 ± 10.1 *vs.* 17.2 ± 2.1 days, $p = 0.0005$, Fig. 4a). Analysis of the capillary density of the tumour revealed a decrease in the number of CD31-positive vessels in CORM-2-treated, CAPAN-2 xenografted animals, compared with mice receiving iCORM-2 ($p < 0.01$, Fig. 4b and c); indicating that CORM-2 was able to substantially affect *de novo* angiogenesis. This finding was consistent with

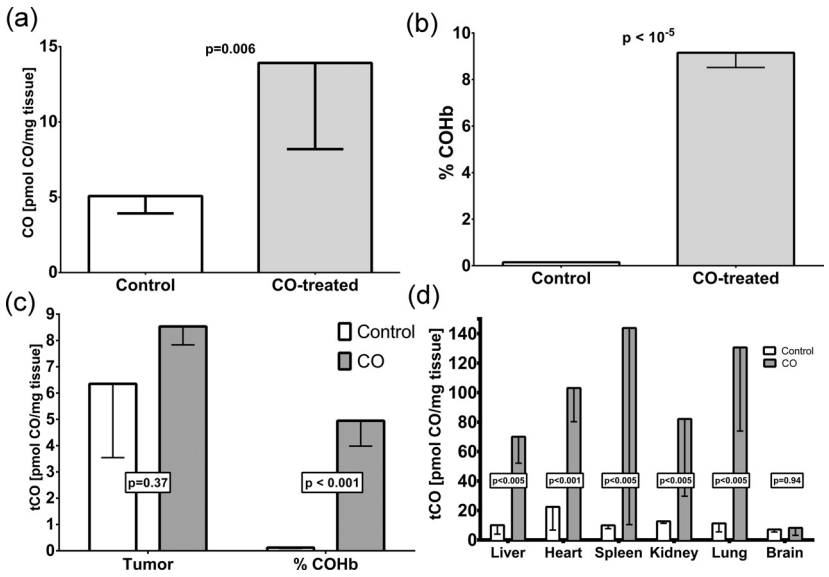

Fig. 5. The effect of CO exposure on its distribution in the body. (a) CO levels in tumour tissues, and (b) CO-haemoglobin levels in animals sacrificed immediately after the last exposure (day 14) to CO (500 ppm CO for 1 h/day). (c) CO levels in tumour tissue and CO-haemoglobin proportion, and (d) CO levels in various other organs of animals sacrificed 8 h after the last exposure (day 14) to CO (500 ppm CO for 1 h/day) control = tissues from animals unexposed to CO; $n = 6$ for each group. CO, carbon monoxide; SD, standard deviation; ppm, parts per million.

our *in vitro* data on CORM-2-induced suppression of Akt phosphorylation, since downstream signals from the Akt signalling pathway are known to contribute significantly to cancer neovascularisation [19].

Similarly, as in the *in vitro* studies, in order to verify whether CO released from the CORM-2 molecule was responsible for the observed antiproliferative/antiangiogenic effects of CORM-2, we also investigated the effect of CO *per se*. We assessed the tumour volume and survival in mice carrying subcutaneous human pancreatic ductal adenocarcinoma PaTu-8902 xenografts, which were treated with CO (500 ppm, 1-h exposure per day). CO-treated PaTu-8902 tumour-bearing mice showed a significant decrease in mean tumour volume ($p < 0.05$, Fig. 4d) and a two-fold increase in survival rate ($p = 0.004$, Fig. 4e), compared with the control mice exposed to air.

Analysis of CO levels in the tumour tissue, performed immediately after the last exposure to CO, demonstrated more than an 170% increase in CO concentrations at target tumours, compared with those of control mice ($p = 0.006$, Fig. 5a); this was paralleled by a marked increase in serum CO-haemoglobin levels ($p < 10^{-5}$, Fig. 5b). To assess the long-term distribution effects of CO exposure, we also assessed tissue levels of CO 8 h after the last exposure to the treatment gas. Consistent with its systemic effects on pancreatic cancer proliferation, CO concentrations in various organs, including subcutaneously xenografted pancreatic cancer, remained substantially high (Fig. 5c) even after this prolonged period of time, indicating the long-term systemic distribution of CO.

4. Discussion

For a long time, CO has been considered a toxic gas with potentially lethal consequences; however, it now appears to have multiple beneficial effects for human health. The importance of CO is underlined by the fact that substantial amounts of this gas is produced endogenously, as a result of the action of HMOX, which is among the most important stress enzymes in the human body [20].

Our *in vitro* and *in vivo* data show that exogenously administered CO, either in the form of CORM-2 or as direct CO exposure, acts as a potent inhibitor of pancreatic carcinogenesis. Indeed, in mice carrying human pancreatic xenografts, CO reduces tumour volume, limits tumour neovascularisation and profoundly prolongs survival. The doses of CO used in our *in vivo* study (500 ppm of CO for 1 h a day) are far below lethal doses for mice, which is equal to inhalation of 2400 ppm for 4 h [21]. This is also evidenced by the acceptable CO-haemoglobin levels of the CO-treated animals, which are even below the CO-haemoglobin concentrations achieved from clinically relevant CO dosages, using a FDA-approved CO inhalation device for human use [7]. Based on the very promising therapeutic potential of CO, the effects of CO inhalation at dosing regimens equivalent to those used in our work are currently being investigated in other clinical trials with patients suffering from pulmonary or intestinal diseases with safety and tolerability issues being investigated as well.[3]

Collectively, our data point to the antiproliferative nature of CO, which may genuinely have a chemotherapeutic/chemoadjuvant potential against certain cancers. CO along with NO and H_2S, belong to the potent bioactive gases having multiple, often shared, biological functions [22]. However, the observed antiproliferative action of CO appears to have the opposite effects to that of NO. NO generated following activation of endothelial nitric oxide synthase (NOS) promotes malignant progression [23] and VEGF-driven neovascularisation [24,25] through activation of the pro-survival Akt pathway [26], which is critical for ischemic and VEGF-mediated angiogenesis [19]. Positive inducible NOS expression correlates with increased microvessel density in human gastric cancer [27], although a dual role of NO in human carcinogenesis has been documented [28]. Based on our data, CO seems to inhibit Akt phosphorylation, which in turn is reflected by decreased neovascularization, as observed in our CORM-2 treated mice. This is in accord with the report by Becker et al. [29], which demonstrated a better prognosis for colon cancer patients overexpressing HMOX1 (responsible for endogenous CO production) in tumour tissues. Furthermore, Ferrando et al. demonstrated that HMOX1 overexpression in prostate cancer cells potently suppressed angiogenesis [30].

[3] www.clinicaltrials.gov [accessed 15.10.13].

Nevertheless, CO seems to contribute to the suppression of carcinogenesis by several additional mechanisms, as shown in the CO-induced inhibition of photocarcinogenesis [31] or migration of hepatoblastoma HepG2 cells [32]. CO derived from CORM-3 was also shown to interfere with the p38 MAPK signalling pathway in endothelial cells [33], which is consistent with our data on the CO-induced inhibition of Akt phosphorylation in pancreatic cancer cells. Furthermore, CO, a potent activator of guanylate cyclase [34], may also modulate cellular proliferation *via* increased cGMP production [35]. Additionally, for the first time we can report pharmacokinetic data of CO inhaled by experimental animals demonstrating a clinically important CO distribution within the various organs and tissues of the mouse body, including subcutaneously xenografted tumours, even after a prolonged period of time. Consistent with these data, we have also validated the efficient distribution of CO within pancreatic cancer cells exposed to the CORM-2 molecule. These kinetic data support the idea that CO might possibly be used in clinical settings.

In conclusion, we found that CO in relatively low (but clinically relevant and applicable) doses acts as a potent anticancer agent. These data suggest that CO could potentially be exploited as a novel chemotherapeutic/chemoadjuvant gas for inhibiting tumour growth.

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Conflict of interest statement

None to report.

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