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PRECLINICAL STUDIES

Enhanced oncolysis mediated by Coxsackievirus A21 in combination with doxorubicin hydrochloride

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Summary Virotherapy is an emerging strategy for the treatment of cancer that utilizes both replication-competent and genetically modified viruses to selectively kill tumor cells. We have previously shown that Coxsackievirus A21 (CVA21), a common-cold producing enterovirus, is an effective oncolytic agent against human melanoma, prostate, and breast cancer xenografts in vivo. CVA21 specifically targets and lytically infects susceptible cells expressing the CVA21 cellular receptors, intercellular adhesion molecule-1 (ICAM-1) and decay-accelerating factor (DAF). Herein, the efficacy of CVA21 administered in combination with doxorubicin hydrochloride as a new therapeutic regimen for cancer was investigated. Flow cytometric analysis demonstrated that the human breast, colorectal, and pancreatic cancer cell lines examined expressed moderate levels of surface ICAM-1 and DAF, whilst a normal breast cell line expressed only minimal levels. When CVA21 was combined with doxorubicin hydrochloride, synergistically enhanced cell death was observed when CVA21 was administered both simultaneously or 24 h prior to doxorubicin hydrochloride exposure. Doxorubicin hydrochloride had no effect on CVA21 replication. Through the use of an orthotopic (MDA-MB-231-luc) xenograft SCID mouse model of human breast cancer we showed that a single intravenous injection of CVA21 in combination with an intraperitoneal injection of doxorubicin hydrochloride resulted in significantly greater tumor reduction compared to either agent alone. Overall, these findings highlight the exciting potential of CVA21, administered in combination with doxorubicin hydrochloride, as a new therapeutic regimen for cancer.

Keywords Coxsackievirus A21 · Oncolytic virus · Breast cancer · Colorectal cancer · Pancreatic cancer · Doxorubicin hydrochloride

Abbreviations

ANOVA	One-way analysis of variance
CI	Combination index
CVA21	Coxsackievirus A21
DAF	Decay-accelerating factor
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
5-FU	5-fluorouracil
HSV-1	Herpes Simplex Virus-1
ICAM-1	Intercellular adhesion molecule-1
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
SCID	Severe combined immunodeficient
SEM	Standard error of the mean
SPF	Specific-pathogen-free
$TCID_{50}$	50% tissue culture infective dose
UV	Ultraviolet

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Introduction

Cancer is one of the leading causes of death worldwide [1], and once it has reached an advanced stage, conventional therapies, such as surgery, chemotherapy, and radiation therapy, have limited success [2, 3]. The development of new therapeutics for advanced cancer are therefore needed.

Viral oncolysis has emerged as a novel anti-cancer treatment involving the use of naturally occurring or genetically modified viruses to selectively target and lyze neoplastic cells [4–6]. One oncolytic virus that is currently under investigation is the wild-type genetically unmodified human enterovirus, Coxsackievirus A21 (CVA21), which induces mild upper respiratory symptoms during natural infection of humans [7–9]. Recently, we have shown that CVA21 is an efficient oncolytic agent that specifically targets and rapidly lyzes human malignant melanoma, multiple myeloma, prostate and breast tumors [10–14] expressing upregulated levels of the CVA21 cellular receptors both in vitro and in vivo. In addition, a Phase I clinical trial in late stage melanoma patients has recently been completed, and has demonstrated that intratumorally administered CVA21 (tradename CAVATAK™) is well tolerated in humans, and that 55.55% of patients experienced stabilization or reduction in injected tumor volumes (Smithers and Shafren, unpublished data).

Despite the fact that many oncolytic viruses are efficacious as single therapies for the treatment of a wide range of cancers in preclinical studies, only one virus has been approved for use in humans as a treatment for cancer [15]. As such, combining oncolytic viral therapy with existing standard anti-cancer treatments may provide a faster route into the clinic. Whilst there have been some instances when combining oncolytic viruses with standard chemotherapeutic agents have decreased the efficacy of either agent administered alone [16, 17], synergistic effects were observed in the vast majority of cases. Adenovirus, when combined with cisplatin and 5-fluorouracil (5-FU) in a mouse model of human ovarian cancer, exhibited enhanced tumor regression [18], and when combined with doxorubicin hydrochloride or cisplatin and 5-FU, completely eliminated liver metastases [19] and non-small cell carcinomas [20]. Similarly, when herpes simplex virus-1 (HSV-1) was combined with cisplatin in a mouse model of head and neck cancer, complete elimination of tumors was observed in 100% of mice, whereas only 14% of cisplatin alone and 42% of HSV-1 treated mice experienced complete regression [21]. HSV-1 combined with cetuximab also enhanced tumor regression compared to either agent alone [22]. In addition, synergistic effects were observed when parvovirus [23] or reovirus [24] were combined with gemcitabine or paclitaxel in pancreatic or non-small cell lung cancer cells, respectively.

As CVA21 rapidly lyzes a variety of cancer cells both in vitro and in vivo [10–14], the effect of combining CVA21 with commonly used chemotherapeutic agents was investigated. Combination of CVA21 and doxorubicin hydrochloride resulted in synergistically enhanced cytotoxicity in human breast, colorectal, and pancreatic cancer cells when compared to that observed with either agent alone. Interestingly, combination of CVA21 with a dose of doxorubicin hydrochloride that alone produced no therapeutic effects in an orthotopic model of human breast cancer caused a dramatic reduction in tumor volumes. Taken together, this data identifies a potentially new treatment regimen involving a genetically unmodified wild-type enterovirus, CVA21, used in conjunction with a widely used chemotherapeutic agent, doxorubicin hydrochloride, for human cancers.

Materials and methods

Cells, viruses, and chemotherapeutic agents

The breast cancer cell lines MDA-MB-231 and T47D, and the pancreatic cancer cell line, PANC-1, were obtained from Dr. R. Sutherland (Garvan Institute of Medical Research, Sydney, Australia). The luciferase expressing MDA-MB-231-luc cells were obtained from Dr. J. Shay (University of Texas Southwestern Medical Centre, Dallas, USA). The colorectal cancer cell line, DLD-1, was obtained from Prof. R. Scott (Medical Genetics, University of Newcastle, Newcastle, Australia). The normal breast cell line, 184A1, was purchased from the American Tissue Culture Collection. Melanoma SK-Mel-28 cells were obtained from Dr. S. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia). All of the breast, colorectal, and pancreatic cancer cell lines were maintained in RPMI 1640 (Invitrogen, Auckland, NZ) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), whilst SK-Mel-28 cells were maintained in DMEM (Invitrogen), supplemented with 2% FBS. 184A1 cells were maintained in MEGM (Cambrex, Mt Waverly, Australia) supplemented with 10% FBS, bovine pituitary extract, hydrocortisone, human epidermal growth factor, and insulin, as per manufacturer's instructions.

The *Kuykendall* prototype strain of Coxsackievirus A21 was obtained from Dr M. Kennett (Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Australia) and was propagated in SK-Mel-28 cells. Stock preparations of CVA21 were purified by centrifugation in 5–30% sucrose gradients as described previously [25]. The peak infectious fractions were pooled, dialyzed against phosphate buffered saline (PBS), and stored at −80°C.

Doxorubicin hydrochloride and 5-fluorouracil (5-FU) were purchased from Sigma (Sydney, Australia), and stock solutions were made in distilled water at a concentration of 10 mg/ml or in dimethyl sulfoxide (DMSO) at a concentration of 2.5 mg/ml, respectively. Paclitaxel and tamoxifen were also purchased from Sigma and 50 mg/ml stock solutions were made in DMSO.

Antibodies

Anti-DAF monoclonal antibody, IH4, which recognizes the third short consensus repeat of DAF [26], was a gift from Dr. B. Loveland (Austin Research Institute, Melbourne, Australia). The anti-ICAM-1 monoclonal antibody, WEHI, specific for the N-terminal domain of ICAM-1 [27], was obtained from Dr. A. Boyd (Queensland Institute for Medical Research, Brisbane, Australia).

Flow cytometry

The surface expression of ICAM-1 and DAF on breast cell lines was determined by flow cytometry. Briefly, 1×10^6 cells were pelleted at 1,000 × g for 5 min and incubated on ice with anti-ICAM-1 or anti-DAF monoclonal antibodies (5 μg/ml diluted in PBS) for 20 min. Cells were then washed with PBS, pelleted at 1,000 × g for 5 min, and resuspended in 100 μl of R-phycoerythin-conjugated F $(ab')_2$ fragment of goat anti-mouse immunoglobulin (DAKO, Sydney, Australia) diluted 1:100 in PBS. Following incubation on ice for 20 min, cells were washed and pelleted as above, resuspended in PBS and analyzed for ICAM-1 and DAF expression using a FACScan analyzer (Becton Dickinson, Sydney, Australia).

In vitro effects of doxorubicin hydrochloride in combination with CVA21 on cell viability

Confluent monolayers of 184A1, MDA-MB-231, T47D, DLD-1 and PANC-1 cell lines in 96-well tissue culture plates were infected with 10-fold serial CVA21 dilutions of stock preparations (100 μl/well in quadruplicate), which were standardized on SK-Mel-28 cells. Either immediately, or 24 h later, doxorubicin hydrochloride was added to the plates at a final concentration of 0.63 μM, 0.32 μM, 0.16 μM, or 0 μM. Following incubation at 37°C/5% CO_2 for 72 h, cell monolayers were microscopically examined and cell viability was determined via MTT assay, as described previously [28]. The data was then quantitatively analyzed for synergy/antagonism using CalcuSyn (Biosoft, Cambridge, England), which is based on the isobologram and combination-index (CI) methods, derived from the median-effect principle of Chou and Talalay. Combinations were defined as synergistic if the CI was less than 0.9, additive if between 0.9 and 1.1, and antagonistic if above 1.1.

Effect of doxorubicin hydrochloride on viral replication

Confluent cell monolayers in 24-well culture plates were inoculated with CVA21 at a multiplicity of infection (MOI) of 10 $TCID_{50}$ (50% tissue culture infective dose)/cell in RPMI 1640 supplemented with 1% FBS, and incubated at 37°C for 1 h. Unbound virus was removed, the wells washed 3 times and overlaid with maintenance media, containing between 0 and 0.63 μM doxorubicin hydrochloride. Following incubation for 0, 2, 4, 6, 8, 10, 24, and 48 h at 37°C, cell suspensions were freeze/thawed once, and the level of infectious CVA21 in cells and supernatant was determined by a plate cell lysis infectivity assay as described above.

Caspase activation following CVA21/doxorubicin hydrochloride treatment

184A1, MDA-MB-231, T47D, DLD-1, and PANC-1 cells were seeded on to a 96-well black plate at 1×10^4 cells/well, and grown overnight at 37°C. Cells were then treated in duplicate with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both. Staurosporine was added to wells as a positive control. Plates were incubated at 37°C for 16 and 24 h, before being assayed for caspase activation using an Apo-ONE® Homogenous Caspase-3/7 assay kit (Promega, Sydney, Australia), as per the manufacture's instructions.

Hoechst stain

184A1, MDA-MB-231, T47D, DLD-1, and PANC-1 cells were seeded onto 96-well plates at 5×10^3 cells/well, and allowed to settle overnight. Cells were then treated with CVA21 (MOI=10 $TCID_{50}$/ml), doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both, and incubated at 37°C for 24 h. Five micrograms of Hoechst stain was added to each well, and incubated at 37°C for 1 h. Apoptosis was determined by examining the cells microscopically using an ultraviolet (UV) filter, and the percentage of apoptotic cells was calculated.

Cell cycle analysis

184A1, MDA-MB-231, T47D, DLD-1, and PANC-1 cells were grown in 6-well plates. Cells were treated with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both for 6 h at 37°C. Cells were harvested, washed once with PBS, and suspended in a 0.1% glucose/PBS and ice-cold

70% ethanol mixture. Twenty-four hours later, the cells were washed once in PBS, and resuspended in 300 μl 50 μg/ml propidium iodide solution that contained 10 mg/ml RNase. Cells were then analyzed using a FACScan analyser (Becton Dickinson).

Orthotopic breast tumor xenograft transplantation in severe combined immunodeficient (SCID) mice

All animal procedures were performed under the guidance and approval of the University of Newcastle Animal Care and Ethics Committee. Specific-pathogen-free (SPF) SCID mice were housed under pathogen-free conditions in the animal handling facility located within the university. Breast cancer cells (MDA-MB-231-luc) were harvested with trypsin, washed once with RPMI 1640, three times with PBS and resuspended in sterile PBS. The viability of cells was assessed by trypan blue staining and only cell preparations with >95% viability were used for xenotransplantation. Tumor cells were xenografted onto the fourth right mammary fat pad of anaesthetized (isofluorane) 6 to 8 week old SCID mice by a single subcutaneous injection of 2×10^6 cells. Once palpable tumors had established, the tumor bearing mice (8 per group) were treated with a single intravenous injection of PBS or CVA21 (1×10^7 $TCID_{50}$), a single intraperitoneal injection of doxorubicin hydrochloride (75 μg/mouse), or a combination of CVA21 and doxorubicin hydrochloride. Xenograft tumor burdens were monitored daily and measured with electronic callipers. Estimates of tumor volumes were calculated using the formula for a spheroid. Mice were monitored over a 23-day period, at which time, the doxorubicin hydrochloride treated mice were sacrificed due to toxicity (weight loss greater than 20% of initial body weight).

Statistical analysis

All in vitro apoptosis experiment statistical analyzes were conducted using one-way analysis of variance (ANOVA) with a Bonferroni post-test, and are presented as the mean± standard error of the mean (SEM). All animal experiment statistical analyzes were conducted using a Student's *t*-test, and are presented as the mean±SEM for the number of mice in each group (n).

Results

Expression of ICAM-1 and DAF on cancer cell lines

We have previously shown that CVA21 is able to rapidly target and lyze a variety of cancer cell types [10–14] expressing high levels of the CVA21 cellular receptors, ICAM-1 and DAF. Herein, the cell surface expression of these receptors was examined in a panel of normal and cancerous breast, colorectal, and pancreatic cell lines. Flow cytometric analysis revealed that all of the cancer cell lines examined possessed abundant levels of surface ICAM-1, whilst the normal cell line (184A1) only expressed minimal surface ICAM-1 (Fig. 1). The two breast cancer cell lines (MDA-MB-231 and T47D) expressed high levels of surface DAF, however, the colorectal (DLD-1) and pancreatic (PANC-1) cancer cell lines, as well as the normal breast line (184A1), expressed only minimal levels of DAF (Fig. 1).

In vitro survival of cells following treatment with a combination of CVA21 and chemotherapeutic agents

When anti-cancer therapies are combined, the absence of antagonistic mechanisms of action need to be confirmed, so that the individual agents can be used sequentially or in tandem in treatment regimens. Four commonly used chemotherapeutic agents (doxorubicin hydrochloride, paclitaxel, tamoxifen, and 5-FU) were combined with CVA21 and the effects on CVA21 replication and cellular lysis were examined in MDA-MB-231 cells (see Supplementary Data). Both paclitaxel and tamoxifen reduced the efficacy of CVA21, with high doses of tamoxifen (10 μM) inhibiting viral replication (see Supplementary Figure 1). Most combinations of paclitaxel or 5-FU and CVA21 were shown to be antagonistic (see Supplementary Table 1). In contrast, doxorubicin hydrochloride failed to exert a detectable impact on viral replication or the efficacy of CVA21-mediated in vitro cell lysis (see Supplementary

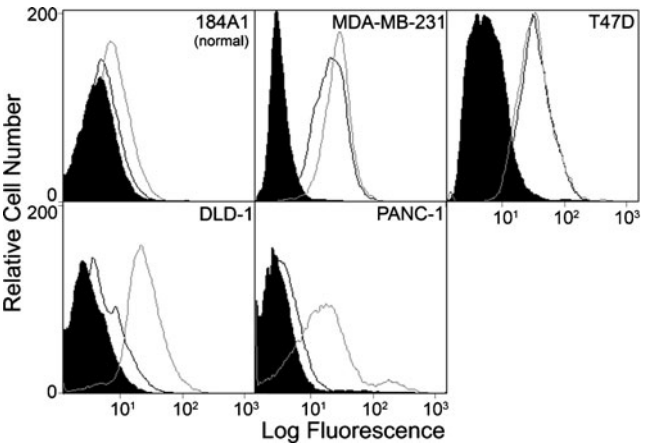

Fig. 1 Flow cytometric analysis of ICAM-1 and DAF expression on cell lines. Cell lines were incubated with either conjugate alone, anti-DAF monoclonal antibody (IH4) or anti-ICAM-1 monoclonal antibody (WEHI). The *filled histograms* represent the binding of the conjugate; the *black solid line histograms* represent ICAM-1 expression; and the *dotted grey line histograms* represent DAF expression

Figure 1), and therefore this chemotherapeutic agent was selected for further experimentation.

When two drugs are administered in combination, the sequence of the treatment regimen needs to be determined; i.e. whether the drugs are best administered simultaneously or if pre-treating with one drug enhances the effects of both. We therefore examined whether administering CVA21 and doxorubicin hydrochloride simultaneously could enhance cell cytotoxicity over the use of either agent alone. Cell monolayers were infected with CVA21 at varying input doses of virus and treated with doxorubicin hydrochloride (0.63 μM, 0.32 μM or 0.16 μM) and incubated at 37°C for 72 h. An MTT assay was then performed, and cell survival and combination indices (CI) were calculated for each cell line and treatment combination. 184A1 cells (Fig. 2a) were not susceptible to CVA21 infection at even the highest dose examined (MOI=10 $TCID_{50}$/cell). Cell death was slightly increased when CVA21 was combined with doxorubicin hydrochloride, however, more than 50% of cells survived these treatments, even at the highest doses examined. The combination of CVA21 and doxorubicin hydrochloride in the 184A1 cells were synergistic for the two highest MOI of CVA21 examined (Table 1), but at the lowest MOI of CVA21 used, these interactions became antagonistic. All combinations of CVA21 and doxorubicin hydrochloride examined in all four cancer cell lines were found to be synergistic (Table 1), suggesting that the use of these two anti-cancer agents increase the efficacy over either agent alone. Combination of CVA21 and doxorubicin hydrochloride in MDA-MB-231 (Fig. 2b), T47D (Fig. 2c), DLD-1 (Fig. 2d), and PANC-1 (Fig. 2e) cells resulted in dramatically increased cell death at all combinations investigated. Therefore, the above findings highlight that simultaneously combining doxorubicin and CVA21 synergistically enhances killing over administration of either agent alone in all cancer cell lines examined.

We next determined whether pre-administering CVA21 for 24 h increased the sensitivity of cell lines to doxorubicin hydrochloride. The sensitivity of 184A1 cells to doxorubicin hydrochloride failed to appear to be altered following exposure to CVA21 for 24 h (Fig. 3a), however, upon examination of the CI values, an antagonistic interaction for most combinations was

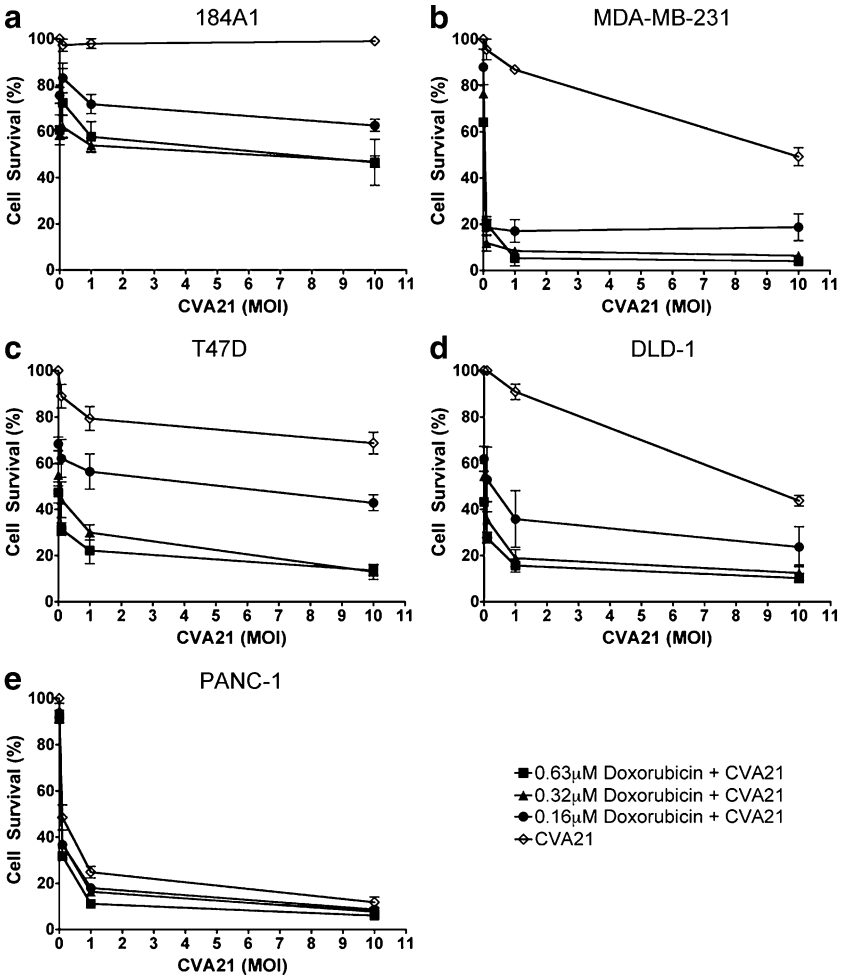

Fig. 2 Cell survival following treatment with a combination of simultaneously administered CVA21 and doxorubicin hydrochloride. Cell monolayers were infected with 10-fold dilutions of a stock preparation of CVA21 [2×10^9 $TCID_{50}$/ml, with viral titers standardized on SK-Mel-28 cells], as well as doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM). Following incubation at 37°C for 72 h, cell survival was determined via MTT assay. Survival of **a** 184A1, **b** MDA-MB-231, **c** T47D, **d** DLD-1, and **e** PANC-1 cells treated with varying concentrations of CVA21 and doxorubicin hydrochloride over a 72 h period. Data is presented as the average ± SEM (n=3)

Table 1 Combination index for cells simultaneously treated with a combination of CVA21 and doxorubicin hydrochloride. Combination indices (CI) were calculated using CalcuSyn, as described in the Materials and methods. CI values less than 0.9 were determined to be synergistic, between 0.9 and 1.1 to be additive, and greater than 1.1 to be antagonistic

Cell Line	CVA21 (MOI)	Doxorubicin Concentration (μM)		
		0.63	0.32	0.16
184A1	10	0.730±0.882	0.225±0.261	0.291±0.181
	1	0.912±1.0	0.310±0.336	0.926±0.365
	0.1	159.2±194.7	0.574±0.427	11.5±8.9
MDA-MB-231	10	0.258±0.160	0.209±0.127	0.393±0.296
	1	0.125±0.086	0.098±0.063	0.080±0.056
	0.1	0.290±0.185	0.089±0.055	0.059±0.041
T47D	10	0.077±0.034	0.043±0.026	0.266±0.033
	1	0.192±0.079	0.173±0.032	0.558±0.158
	0.1	0.390±0.115	0.484±0.150	0.856±0.357
DLD-1	10	0.433±0.143	0.474±0.167	0.807±0.371
	1	0.185±0.119	0.152±0.078	0.342±0.191
	0.1	0.317±0.227	0.360±0.222	0.712±0.435
PANC-1	10	0.312±0.087	0.489±0.166	0.585±0.183
	1	0.237±0.098	0.369±0.071	0.427±0.105
	0.1	0.440±0.140	0.509±0.105	0.444±0.116

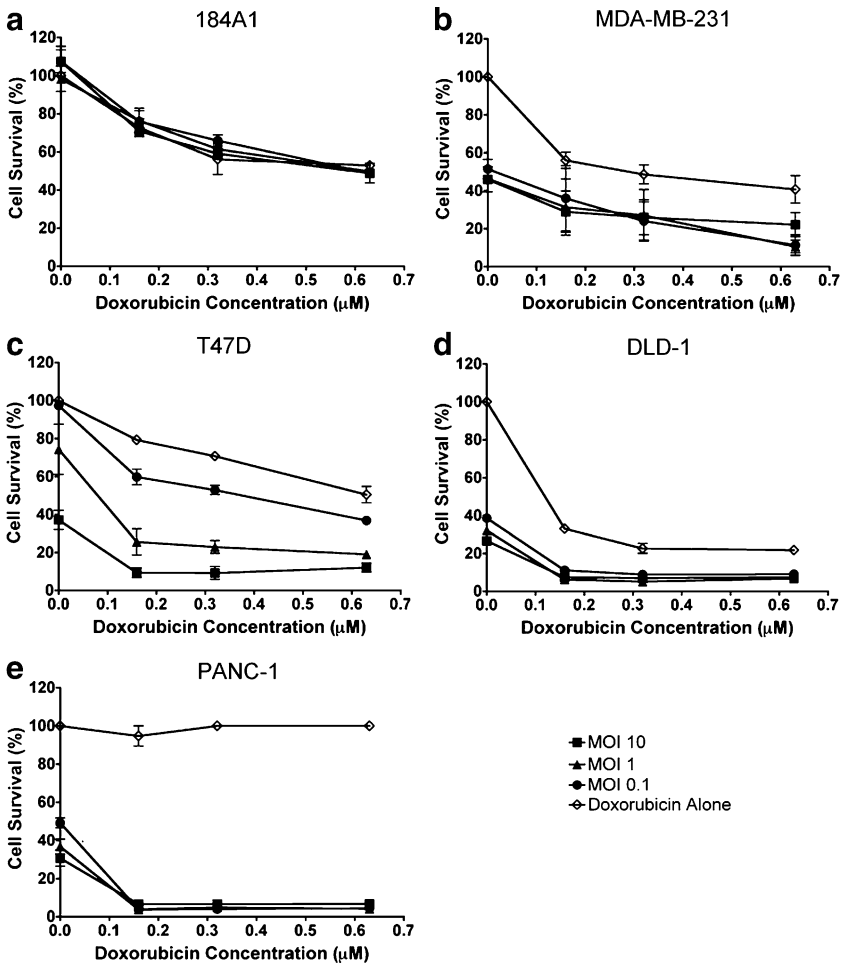

Fig. 3 Effect of CVA21 administered prior to treatment with doxorubicin hydrochloride on cell survival. Cell monolayers were infected with 10-fold dilutions of a stock preparation of CVA21 [2×10^9 $TCID_{50}$/ml, with viral titers standardized on SK-Mel-28 cells]. Twenty-four hours later, doxorubicin hydrochloride was added at varying concentrations (0.63 μM, 0.32 μM, or 0.16 μM). Following incubation at 37°C for 72 h, cell survival was determined via MTT assay. Survival of **a** 184A1, **b** MDA-MB-231, **c** T47D, **d** DLD-1, and **e** PANC-1 cells treated with varying concentrations of CVA21 and doxorubicin hydrochloride over a 72 h period. Data is presented as average ± SEM (n=3)

Table 2 Combination index for cells treated with CVA21, then 24 h later treated with doxorubicin hydrochloride. Combination indices (CI) were calculated using CalcuSyn, as described in the Materials and methods. CI values less than 0.9 were determined to be synergistic, between 0.9 and 1.1 to be additive, and greater than 1.1 to be antagonistic

Cell Line	CVA21 (MOI)	Doxorubicin Concentration (μM)		
		0.63	0.32	0.16
184A1	10	1.1±0.2	1.8±1.3	2.2±1.7
	1	1.4±0.5	2.5±2.0	1.2±0.4
	0.1	0.967±0.122	3.9±3.2	1.0±0.3
MDA-MB-231	10	0.206±0.209	0.409±0.316	1.1±0.8
	1	0.041±0.034	0.574±0.668	1.0±1.1
	0.1	0.090±0.077	0.105±0.077	1.2±1.4
T47D	10	0.397±0.138	0.222±0.067	0.256±0.158
	1	0.265±0.072	0.190±0.042	0.148±0.059
	0.1	0.558±0.188	0.561±0.149	0.403±0.107
DLD-1	10	0.078±0.054	0.035±0.023	0.020±0.014
	1	0.059±0.036	0.019±0.015	0.016±0.012
	0.1	0.120±0.078	0.058±0.034	0.052±0.034
PANC-1	10	0.256±0.238	0.133±0.125	0.065±0.061
	1	0.221±0.205	0.122±0.116	0.059±0.057
	0.1	0.245±0.238	0.124±0.121	0.059±0.057

observed (Table 2). The sensitivity of MDA-MB-231 cells to doxorubicin hydrochloride was greatly enhanced following exposure to CVA21 (Fig. 3b). Upon examination of the CI values, synergism was noted for the two highest concentrations of doxorubicin hydrochloride examined (0.63 μM and 0.32 μM doxorubicin hydrochloride), however, an antagonistic response was noted for the lowest dose of doxorubicin hydrochloride (0.16 μM doxorubicin hydrochloride) (Table 2). The cytotoxicity of doxorubicin hydrochloride was synergistically enhanced (Table 2) in T47D (Fig. 3c), DLD-1 (Fig. 3d), and PANC-1 (Fig. 3e) cells. Taken together, our data demonstrates that in general, pre-administering CVA21 synergistically enhances the cytotoxicity of doxorubicin hydrochloride in cancer cells, with no enhancement in normal breast cells.

Doxorubicin hydrochloride has no effect on CVA21 replication

To confirm that doxorubicin hydrochloride exposure is not able to inhibit CVA21 replication, one-step growth curve studies were undertaken. Normal and cancer cell lines were challenged with CVA21 (MOI=10 $TCID_{50}$/cell), and the production of progeny virus was monitored over a 48 h period. CVA21 underwent multi-cycle replication in all cell lines, with the normal breast line, 184A1, exhibiting the lowest level of viral amplification (Fig. 4a). No difference in CVA21 replication was observed in the 184A1 (Fig. 4a), MDA-MB-231 (Fig. 4b), T47D (Fig. 4c), or PANC-1 (Fig. 4e) cell lines when conducted in the presence of doxorubicin hydrochloride. However, replication was slightly decreased in DLD-1 cells treated with doxorubicin hydrochloride (Fig. 4d) at 48 h post-infection, which is potentially due to the rapid cell death observed following the combination of doxorubicin hydrochloride and CVA21, compared to cells infected only with CVA21.

Mechanism behind enhanced cytotoxicity in cells treated with a combination of CVA21 and doxorubicin hydrochloride

To determine the mechanism behind the synergistic relationship between CVA21 and doxorubicin hydrochloride, the effect of doxorubicin hydrochloride on the level of surface expression of CVA21 cellular receptors was examined. Cells were treated with doxorubicin hydrochloride for 24 h, and then analyzed via flow cytometry for changes in surface expression of ICAM-1 and DAF. Surface levels of ICAM-1 and DAF remained unchanged following doxorubicin hydrochloride treatment (data not shown), indicating that the enhanced cell killing observed is most likely not due to changes in receptor expression.

Next, differences in apoptotic induction between cells treated with CVA21, doxorubicin hydrochloride, or a combination of both was examined. Caspase activation 16 and 24 h following treatment was measured using the Apo-ONE® Homogenous Caspase-3/7 assay kit. As expected, there was an increase in active caspase levels at 24 h (Fig. 5b) compared to 16 h (Fig. 5a). Caspase levels at 16 h (Fig. 5a) and 24 h (Fig. 5b) post-treatment were significantly greater in the combination treated cancer cells than

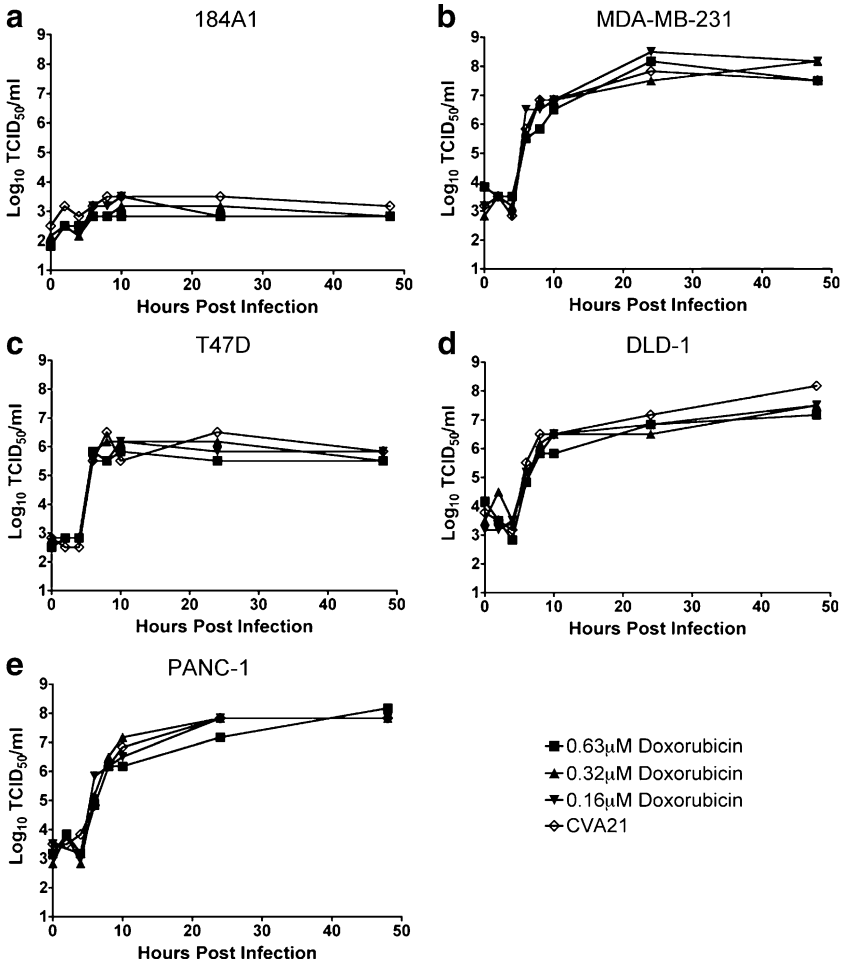

Fig. 4 Effect of doxorubicin hydrochloride on CVA21 replication. Cell monolayers were inoculated with CVA21 (MOI= 10 $TCID_{50}$/cell). Following the removal of unbound virus, the cell monolayers were incubated with 0.63 μM, 0.32 μM, 0.16 μM, or 0 μM doxorubicin hydrochloride for 48 h. At selected time points (0, 2, 4, 6, 8, 10, 24, and 48 h), cell suspensions were freeze-thawed, clarified, and virus yield was determined by viral lytic assay on SK-Mel-28 cells (n=3)

in those that were treated only with doxorubicin hydrochloride. The levels of caspase induction in the MDA-MB-231 and PANC-1 cell lines were further increased in the combination samples when compared to CVA21 alone. However, this increase was not observed in T47D and DLD-1 cells. Only minimal caspase induction was observed in the normal breast cell line, 184A1.

Next, the total number of apoptotic cells following 24 h of treatment with CVA21 and/or doxorubicin hydrochloride was examined by Hoechst stain. Once again, low levels of apoptotic induction were observed in the 184A1 cells (Fig. 6a). However, in the MDA-MB-231 (Fig. 6b) and DLD-1 (Fig. 6d) cells, a greater number of apoptotic cells was noted in the combination treated samples compared to either the doxorubicin hydrochloride or CVA21 alone samples, indicating that the enhanced cell killing may be due to increased levels of apoptotic induction. However, in the T47D (Fig. 6c) and PANC-1 (Fig. 6e) cells, the percentage of apoptotic cells, whilst being greater in combination treated versus doxorubicin hydrochloride only treated cells, was similar to that observed following CVA21 treatment alone, indicating that different mechanisms of cell death may be occurring in these cells compared to MDA-MB-231 and DLD-1 cells.

Cell cycle analysis of cells treated with a combination of doxorubicin and CVA21

Whilst relatively little is known concerning the effect of CVA21 infection on the cell cycle, doxorubicin hydrochloride is known to cause an arrest at G2/M [29], thereby inhibiting mitotic progression and causing cell death. To gain mechanistic insight into the enhanced apoptotic cell death induced by combining CVA21 and doxorubicin hydrochloride, we investigated the action of these two agents on cell cycle progression. Cells were treated with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both for 6 h, and cell cycle stage was analyzed by flow cytometry. The percentage of cells in each phase of the cell cycle was calculated. Neither CVA21 or doxorubicin hydrochloride treatment exerted a detectable effect on the cell cycle of 184A1 cells (Fig. 7a), whereas MDA-MB-231 (Fig. 7b), T47D (Fig. 7c), DLD-1 (Fig. 7d), and PANC-1

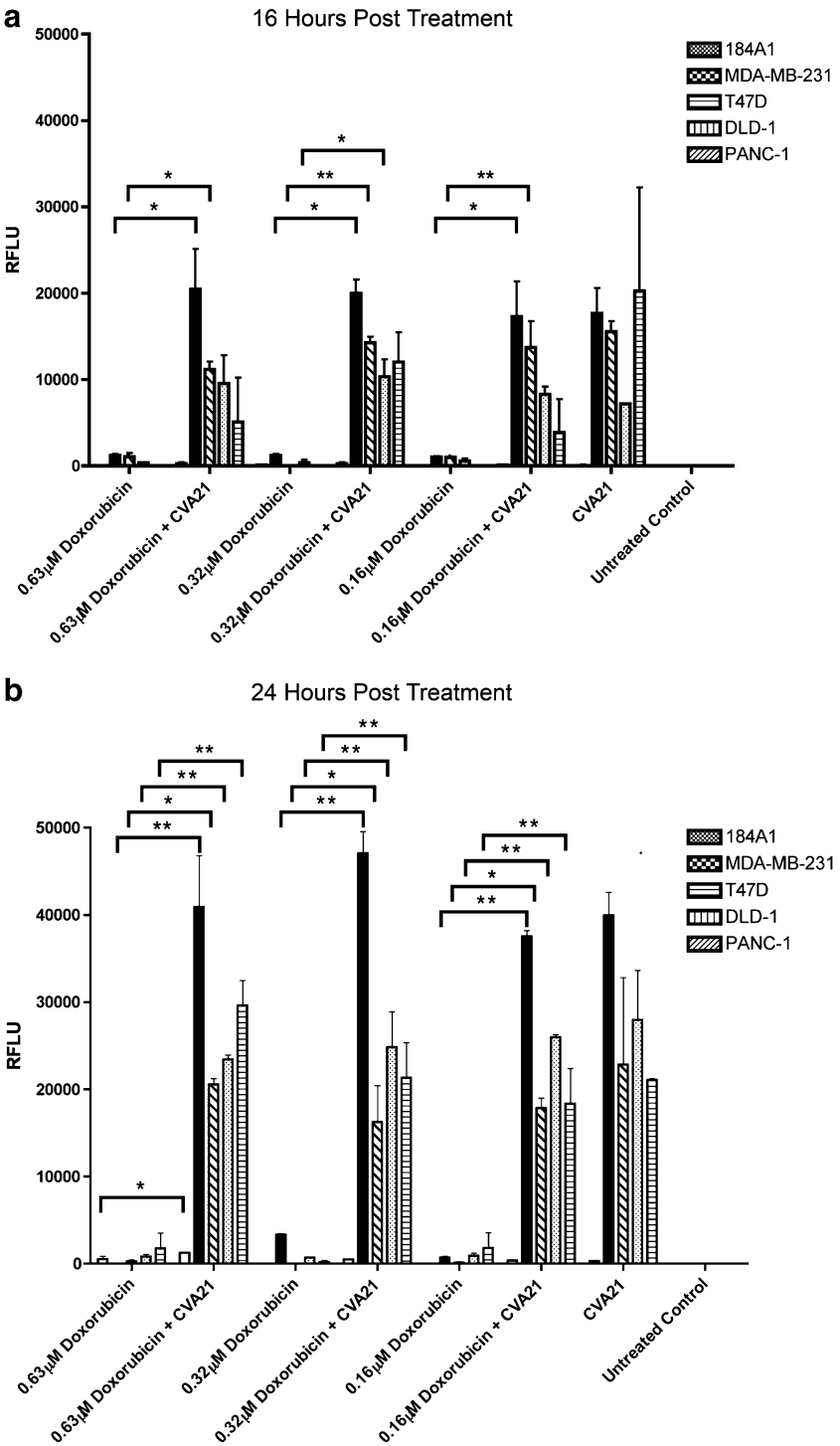

Fig. 5 Caspase activation following treatment with CVA21 and/or doxorubicin hydrochloride. Cells were treated with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both. At selected time points, caspase activation was examined via the Apo-ONE® Homogenous Caspase-3/7 Assay Kit (Promega). Caspase activation at **a** 16 h and **b** 24 h post-treatment. * denotes statistical significance ($p<0.05$; $n=3$); ** statistical significance ($p<0.01$; $n=3$)

(Fig. 7e) cells treated with doxorubicin hydrochloride were found to predominantly be in G2/M phase. Infection with CVA21 resulted in an accumulation of cells in the S, or 'synthesis' phase (Fig. 7) of the cell cycle. The combination of CVA21 and doxorubicin hydrochloride increased the percentage of apoptotic cells in the cancer cell population, thereby confirming the caspase and Hoechst stain findings (Figs. 5 and 6).

In vivo combination of CVA21 and doxorubicin hydrochloride

The in vitro data presented herein demonstrated that CVA21, when combined with doxorubicin hydrochloride, increased cell death over the activity of either agent alone. To assess the combinatorial effects in vivo, SCID mice with pre-established MDA-MB-231-luc tumor xen-

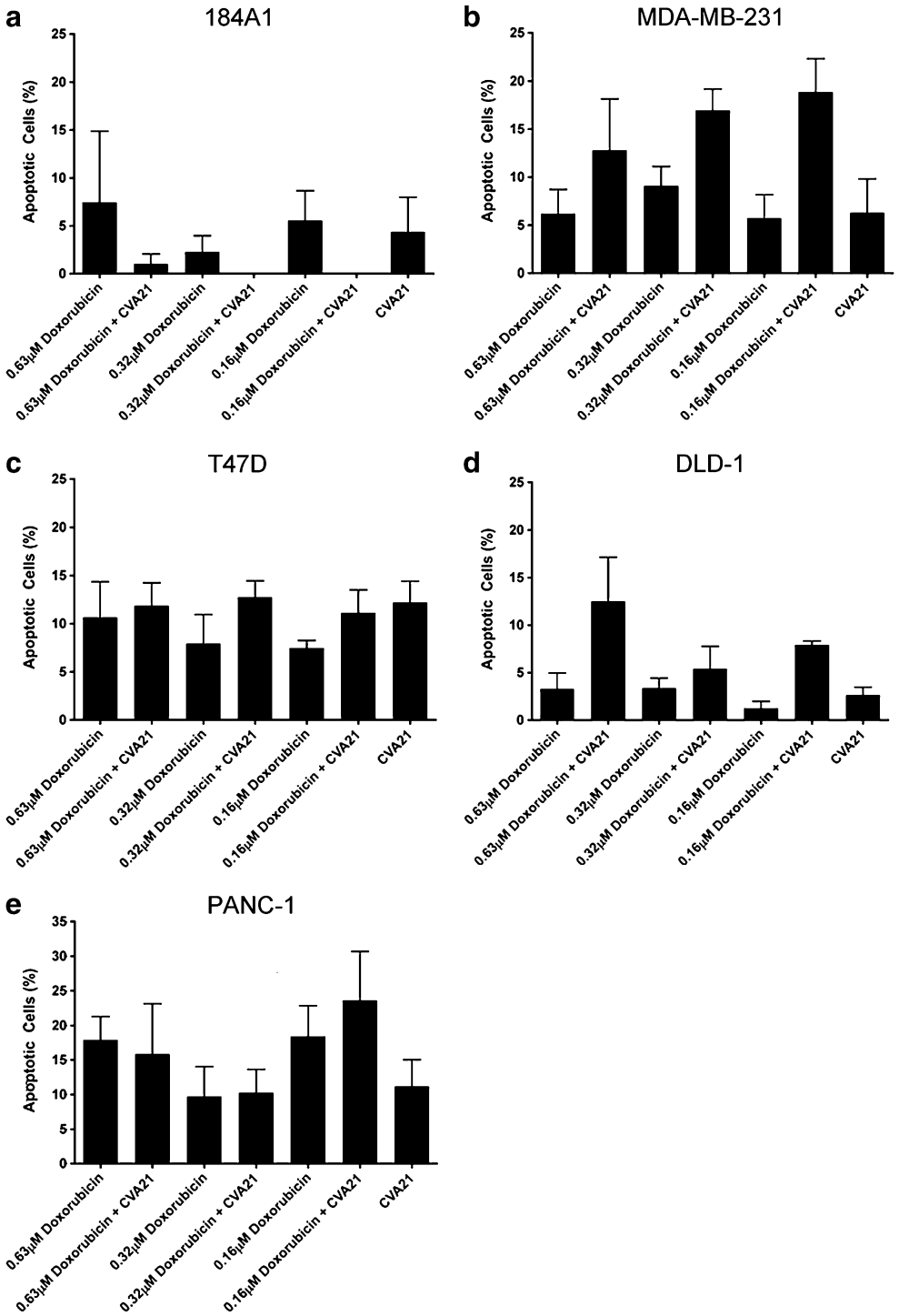

Fig. 6 Apoptosis in cancer cells 24 h post-treatment with CVA21 and/or doxorubicin hydrochloride. Monolayers of **a** 184A1, **b** MDA-MB-231, **c** T47D, **d** DLD-1, and **e** PANC-1 cells were plated in 96-well plates. Cells with treated with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin (0.63 μM, 0.32 μM, or 0.16 μM), or a combination of both. Twenty-four hours later, cells were incubated with 5 μg of Hoechst stain for 1 h at 37°C, and then microscopically examined for the presence of apoptotic bodies using a UV-filter. Apoptosis was quantified as percentage of apoptotic cells, with n=3. Samples were standardized against the non-treated control

ografts were administered a single intravenous injection of PBS or CVA21 (1×10^7 $TCID_{50}$), a single intraperitoneal injection of doxorubicin hydrochloride (75 μg), or a single intravenous injection of CVA21 plus an intraperitoneal injection of doxorubicin hydrochloride. The doxorubicin hydrochloride dose administered was subtherapeutic. Mouse tumor burdens were measured over a 23-day period (Fig. 8). During the observation period, CVA21 administration significantly ($p<0.05$) reduced the tumor size of mice when compared to those of the PBS treated controls. Tumor volumes in the doxorubicin hydrochloride plus CVA21-treated group were significantly reduced ($p<0.05$) compared to those observed in the PBS-treated mice from day 7 onwards, and were also significantly diminished when compared to both CVA21 ($p<0.04$) and doxorubicin hydrochloride ($p<0.03$) alone.

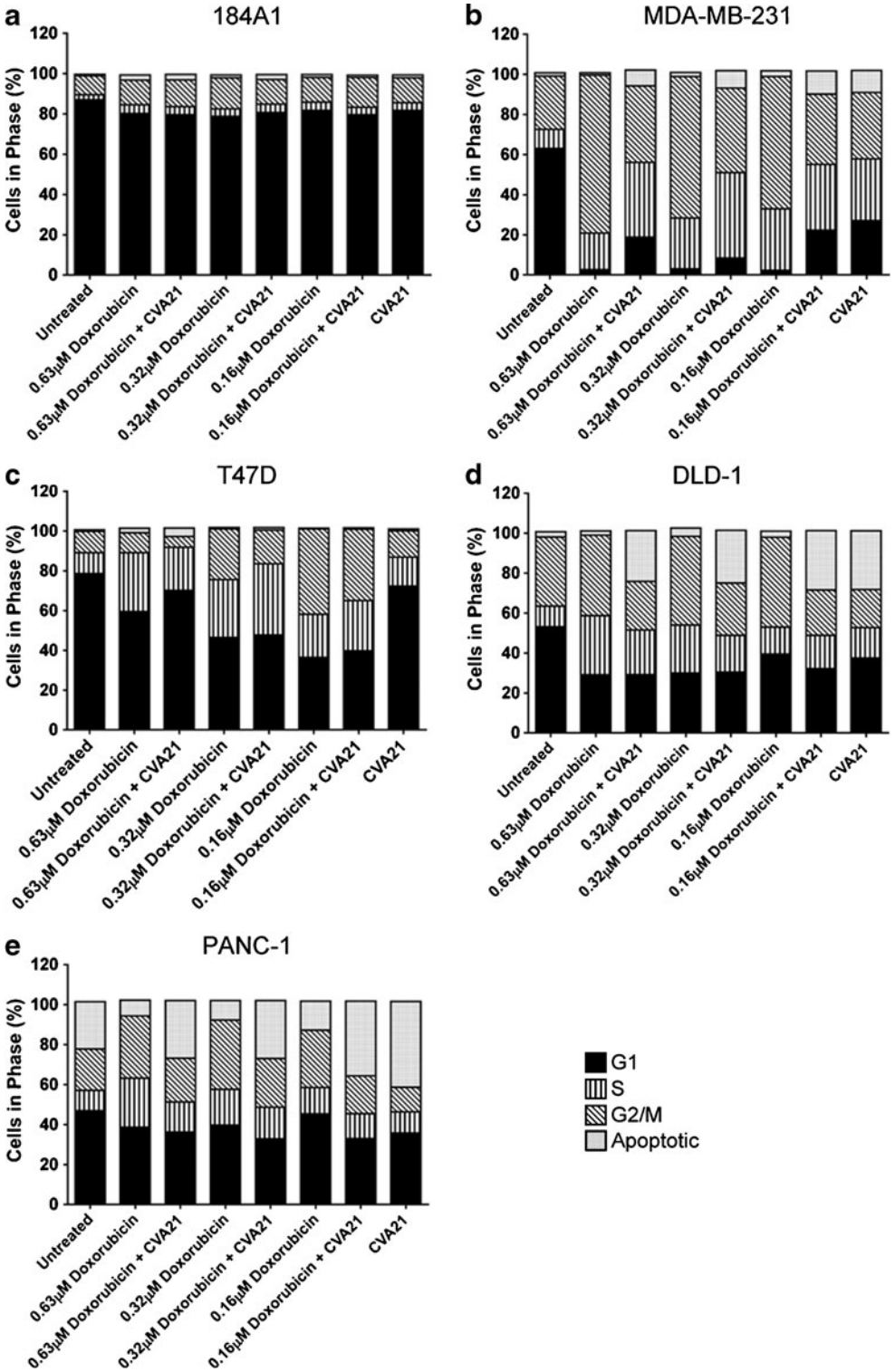

Fig. 7 Cell cycle analysis in cells treated with CVA21 and/or doxorubicin hydrochloride. Cell monolayers were treated with CVA21 (MOI=10 $TCID_{50}$/cell), doxorubicin hydrochloride (0.63 μM, 0.32 μM, or 0.16 μM) or a combination of both for 6 h at 37°C. Cells were then harvested, washed once in PBS, and resuspended in a 0.1% glucose/PBS and ice-cold 70% ethanol solution. Twenty-four hours later, cells were washed, and resuspended in a propidium iodide solution (with RNase). Cell cycle profiles were then examined via flow cytometry. Results are presented as percentage of cells in each phase of the cell cycle for **a** 184A1, **b** MDA-MB-231, **c** T47D, **d** DLD-1, and **e** PANC-1 cells. Representative data from three independent experiments

For ethical reasons, all animals in the doxorubicin hydrochloride alone treatment group were euthanized 13 days post-treatment due to excessive weight loss (greater than 20%), whereas 50% of the CVA21 plus doxorubicin hydrochloride treated mice remained within the ethical limits at this time point. Twenty-five percent of the combination treated mice were still within weight limits at day 23 post-treatment. In this environment, the above findings demonstrate that combining CVA21 with doxorubicin hydrochloride in vivo results in significantly enhanced tumor reduction over that observed following treatment with either agent alone.

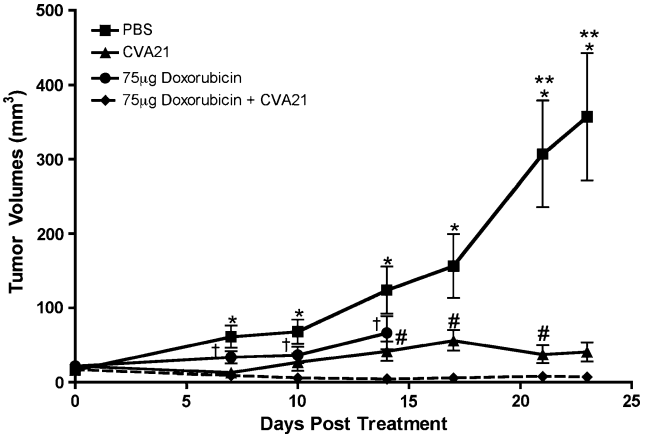

Fig. 8 Oncolytic activity of CVA21 in combination with doxorubicin hydrochloride in a SCID mouse model of human breast cancer. Severe combined immune-deficient (SCID) mice were injected into the fourth right mammary fat pad with MDA-MB-231-luc cells, and tumors were allowed to form before mice were treated with a single intravenous injection of either PBS or CVA21 (1×10^7 $TCID_{50}$), a single intraperitoneal injection of doxorubicin hydrochloride (75 μg), or a combination of CVA21 and doxorubicin hydrochloride. All tumor volumes are expressed as the average tumor burdens ± SEM (standard error of the mean, $n=8$). * PBS vs. CVA21 + doxorubicin hydrochloride statistical significance ($p<0.05$); ** PBS vs. CVA21 statistical significant ($p < 0.05$); † doxorubicin hydrochloride vs. CVA21 + doxorubicin hydrochloride statistical significance ($p<0.03$); # CVA21 vs. CVA21 + doxorubicin hydrochloride statistical significance $p<0.04$)

Discussion

At present, advanced cancer is thought to be incurable, with current treatments, such as chemotherapy and radiotherapy, producing many deleterious side effects. As such, novel therapies that are able to selectively kill cancer cells need to be developed. One such therapy is virotherapy. The effects of virotherapy can be enhanced via combination with various chemotherapeutic agents [18, 19, 21]. In this study, the effect of combining doxorubicin hydrochloride and CVA21 as a potential method of treating breast, colorectal, and pancreatic cancers was examined. Synergistically enhanced cytotoxicity was observed both in vitro and in vivo following a single systemic (intravenous) dose of CVA21 in combination with a single intraperitoneal dose of doxorubicin.

One of the most widely used therapies for metastatic breast cancer is doxorubicin hydrochloride. Doxorubicin hydrochloride can also be used in combination with other chemotherapeutics as a therapy for late stage colorectal [30] and pancreatic [31] cancers. As such, the effect of CVA21 when combined with doxorubicin hydrochloride on breast, colorectal, and pancreatic cancer cells was examined. Previously, additive or synergistic relationships between oncolytic viruses and various chemotherapeutic agents have been observed [18, 19, 32, 33]. Combination of CVA21 and doxorubicin hydrochloride, both when administered simultaneously (Fig. 2) and when CVA21 was pre-administered (Fig. 3), synergistically enhanced cell death in vitro in all cancer cell lines examined (Tables 1 and 2). In addition, doxorubicin hydrochloride failed to effect CVA21 replication in vitro (Fig. 4). Such results are interesting, as they indicate that CVA21 and doxorubicin hydrochloride could potentially be used in combination as a first-line therapy for neoplastic disease. Alternatively, CVA21 could be used as a sensitising agent for treatment with doxorubicin hydrochloride, potentially resulting in a lower dose of chemotherapeutic agent being needed, resulting in a decrease in the side effects observed following doxorubicin hydrochloride administration.

Levels of apoptotic induction were enhanced in MDA-MB-231 (Fig. 6b) and DLD-1 (Fig. 6d) cancer cells treated with a combination of doxorubicin hydrochloride and CVA21 over those treated with either agent alone. Apoptotic levels were enhanced in T47D (Fig. 6c) and PANC-1 (Fig. 6e) cells treated with both CVA21 and doxorubicin hydrochloride compared to doxorubicin hydrochloride alone, and were at a level similar to that observed in CVA21 only treated cells. Therefore, the enhanced cell death following combination with doxorubicin hydrochloride and CVA21 may be due to increased levels of apoptosis. The enhanced cell death following combination of CVA21 and doxorubicin in 184A1 cells (Figs. 2a and 3a) were not as pronounced as that observed in the cancer cell lines (Figs. 2 and 3), suggesting that normal cells may not be as susceptible as cancer cells to the toxic effects of combinatorial therapies. Taken together, our data highlights the potential application of combining CVA21 and doxorubicin hydrochloride as a selective treatment for cancer.

When doxorubicin hydrochloride and CVA21 were combined in vivo in an orthotopic mouse model of human breast cancer, a rapid reduction in tumor volume was observed (Fig. 8). Such tumor reduction was significantly greater than that observed for either agent alone. Overall, the above findings demonstrate the enhancement of an already potent potential therapy for human breast cancer, and confirm the exciting application of combinatorial therapies involving novel oncolytic viruses.

Doxorubicin hydrochloride, whilst being an effective anti-cancer agent, like many chemotherapeutics has several severe side effects. These include neutropenia, thrombocytopenia, anaemia, and cardiac toxicity [34–36]. Interestingly, the dose of doxorubicin hydrochloride used in this study (Fig. 8) exhibited no therapeutic effects, despite causing severe side effects, when administered as a single agent. However, a significantly enhanced tumoricidal effect was observed when this low dose was administered in combination with CVA21, thereby indicating that lower doses of toxic chemotherapeutic agents may be administered to

patients in combination with CVA21, with enhanced efficacy and reduced toxicity.

In conclusion, we have demonstrated the capacity of CVA21, both alone and in combination with doxorubicin hydrochloride, to effectively and potently target and lyze human breast, colorectal, and pancreatic cancer cells both in vitro and in vivo within a mouse xenograft model of human breast cancer. Combination of CVA21 with a standard chemotherapeutic agent, doxorubicin hydrochloride, led to increased tumor cell death in vitro, and enhanced tumor regression in vivo over either agent alone. Non-genetically modified wild-type CVA21 has several major advantages as an oncolytic agent including its mild pathogenicity in humans, and its rapid and efficient lytic infection of cancer cells. Furthermore, additional positive attributes lie in the capacity of CVA21 to amplify low viral input doses that have the potential to spread to distant sites, and a low prevalence of neutralizing antibodies present in the community [10]. Taken together, this data highlights the potential that CVA21, either singly or in combination with doxorubicin hydrochloride, possesses in delivering a new approach for the treatment of cancer.

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Declaration of competing interests Darren Shafren and Richard Barry hold shares in Viralytics Ltd and are currently conducting research sponsored by this company.

Authors' contributions KS participated in the design of the study, carried out the in vitro and in vivo work, and drafted the manuscript. RB participated in the design of the study. DS participated in the design of the study, and was involved in the revision of the manuscript. All authors approved the final manuscript.

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