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ORIGINAL ARTICLE

# Cell cycle and apoptosis regulatory protein (CARP)-1 is a novel, adriamycin-inducible, diffuse large B-cell lymphoma (DLBL) growth suppressor

**Edi Levi · Liyue Zhang · Amro Aboukameel · Sunny Rishi · Ramzi M. Mohammad · Lisa Polin · James S. Hatfield · Arun K. Rishi**

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**Abstract** Diffuse large B-cell lymphoma (DLCL) accounts for 30–40% of adult non-Hodgkin's Lymphoma (NHL). Current anti-NHL therapies often target cellular growth suppression pathways and include R-CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone plus monoclonal anti-CD20 antibody rituximab). However, since many patients relapse, resistant cells to these therapies remain a significant problem and necessitate development of new intervention strategies. Cell cycle and apoptosis regulatory protein (CARP)-1 functions in a biphasic manner to regulate growth factor as well as chemotherapy (adriamycin, etoposide, or iressa)-dependent signaling.

*Purpose* To determine whether CARP-1 is a novel suppressor of lymphoma growth.

*Methods* Flow cytometric analyses coupled with Western immunoblotting, cell growth, apoptosis, and immunocytochemistry methodologies were utilized to determine CARP-1-dependent lymphoma growth inhibition in vitro and in vivo.

*Results* CARP-1 expression correlated with activated caspase-3 and inversely correlated with activated Akt in DLCL. Exposure to adriamycin stimulated CARP-1 expression and inhibited growth of Raji cells, but not CHOP-resistant WSU-DLCL2 cells. Expression of wild-type CARP-1 or its apoptosis-inducing mutants inhibited growth of Raji as well as CHOP-resistant WSU-DLCL2 cells, in part by activating caspase-9 and apoptosis. Since CARP-1 harbors multiple, apoptosis-promoting subdomains, we investigated whether epigenetic compensation of CARP-1 function by intracellular delivery of trans-activator of transcription (TAT) domain-tagged CARP-1 peptide(s) will inhibit lymphoma growth. Treatments with TAT-tagged CARP-1 peptides suppressed growth of the Raji and WSU-DLCL2 cells by stimulating apoptosis. TAT-CARP-1 (1-198) as well as (896-1150) peptides also suppressed growth of WSU-DLCL2 cell-derived tumor xenografts in SCID mice, while administration of TAT-CARP-1 (1-198) also inhibited growth of WSU-FSCCL cell-derived ascites and prolonged host survival.

*Conclusion* CARP-1 is a suppressor of NHL growth and could be exploited for targeting the resistant DLCL.

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E. Levi · L. Zhang · S. Rishi · J. S. Hatfield · A. K. Rishi (✉)
Room B4334, Veterans Affairs Medical Center,
Wayne State University, 4646 John R.,
Detroit, MI 48201, USA
e-mail: Rishia@Karmanos.org

A. Aboukameel · R. M. Mohammad · L. Polin · A. K. Rishi
Department of Internal Medicine,
Wayne State University,
Detroit, MI 48201, USA

E. Levi
Department of Pathology,
Wayne State University,
Detroit, MI 48201, USA

S. Rishi · L. Polin · A. K. Rishi
Karmanos Cancer Institute,
Wayne State University, Detroit, MI 48201, USA

*Present Address:*
L. Zhang
Department of Obstetrics & Gynecology, and Biochemistry,
University of Western Ontario Schulich School of Medicine
and Dentistry, Victoria Research Labs, A4-130a,
800 Commissioners Road East, London, ON, Canada

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## Introduction

Programmed cell death (apoptosis) regulates diverse aspects of animal physiology that include fetal development, cellular homeostasis, immune cell education, and aging. Accumulating evidence suggests a linkage between pathogenesis of multiple diseases and defects in regulation of apoptosis. The pathways regulating apoptosis, therefore, serve as important targets for many anticancer agents currently utilized for treatment of diverse malignancies [1]. CARP-1 (cell cycle and apoptosis regulatory protein; aka CCAR1) is a novel perinuclear phosphoprotein that regulates apoptosis by adriamycin or etoposide [2, 3]. In addition to being a key transcriptional co-activator of p53 in regulating DNA damage–induced apoptosis, CARP-1 regulates growth signaling by the steroid/thyroid receptor superfamily of transcriptional factors as well as $\beta$-catenin [3, 4]. Exposure to adriamycin results in increased expression of CARP-1, while loss of CARP-1 inhibits apoptosis by adriamycin but not Cisplatin, suggesting that CARP-1 is a specific and important mediator of signaling by adriamycin or etoposide [2, 3]. CARP-1 has multiple, non-overlapping, growth inhibitory subdomains [5]. CARP-1-dependent cell growth inhibition involves down-regulation of various cell cycle- and proliferation-related genes, such as p21Rac1, c-Myc, cyclin B1, and topoisomerase II$\alpha$, induction of CDKI $p21^{WAF1/CIP1}$, and activation of caspases 9 and 3 [2, 5].

Diffuse large B-cell lymphoma (DLBL/DLCL) is a heterogeneous lymphoid neoplasm that accounts for 30–40% of adult NHL and has variable clinical outcome, genetic features, and cells of origin [6, 7]. Currently used therapeutic regimens for DLBL include cyclophosphamide, adriamycin, vincristine, and prednisone in combination with anti-CD20 monoclonal antibody rituximab (R-CHOP)-based chemotherapy, while for high-risk subset of DLBLs, increased doses of conventional chemotherapy combined with additional stem-cell support are employed [8, 9]. In order to develop a comprehensive molecular approach to DLBL outcome prediction, recently DNA microarray-based studies were conducted to obtain gene-expression profiling to extend current biological insights into the disease. In one study, cDNA microarrays enriched in genes related to the germinal centers (GC) of the lymphoid organs were used to obtain the expression patterns of DLBL and normal lymphocytes including in vitro-activated peripheral blood (PB) B cells. This investigation found that GC-like DLBLs have more favorable outcome compared with PB-like DLBLs [8]. Additional investigation employing oligonucleotide microarray-based expression profiling also implicated multiple, critical genes in predicting DLBL outcome [9]. The genes implicated in DLBL outcome involved those regulating responses to B-cell-receptor signaling, serine/threonine phosphorylation pathways, and apoptosis [9]. However, development of resistance to anti-NHL therapeutics continues to be a problem, in part, because the precise mechanism(s) of action of anti-lymphoma agents such as adriamycin remain to be clarified and, therefore, warrants investigation of new intervention strategies.

Development and progression of many cancers including NHL is, in part, influenced by loss of function and/or expression of tumor suppressor(s). Epigenetic compensation of tumor suppressor function is therefore an attractive and emerging anti-cancer modality. The trans-activator of transcription (TAT) domain-tagged peptide(s) derived from tumor suppressors such as p53 and the von Hippel–Lindau proteins have shown promise as anti-cancer agents [10–12]. In this context, we previously found that TAT-tagged CARP-1 peptides inhibited growth of the human breast cancer (HBC) cells in vitro as well as suppressed growth of HBC cell-derived xenografted tumors in immunocompromised mice [13].

While it is evident that CARP-1 is an important transducer of cell growth signaling, it remains to be fully understood as to how CARP-1 transduces adriamycin-dependent growth suppression. Knowledge of growth suppression mechanisms by CARP-1 will likely complement strategies for effective targeting of DLBL by therapeutics such as adriamycin. Since CARP-1 has cell growth inhibitory and apoptosis-promoting properties, here we investigated the role of CARP-1 in lymphoma biology and its correlation with apoptosis and the relation between adriamycin responses. Microarray analysis of DLBLs revealed a correlation between the expression of CARP-1 and the apoptotic index as measured by the levels of activated mediators of proliferation and apoptosis, such as Akt and caspase 3, respectively. In vitro experiments revealed NHL cell growth inhibitory properties of the wild-type CARP-1 as well as its mutants. Furthermore, we found that affinity-purified TAT-tagged CARP-1 peptides were effective suppressors of NHL growth in vivo, and CARP-1-dependent NHL growth inhibition in vitro and in vivo involved elevated apoptosis.

## Materials and methods

### Materials

DMEM, Ham's F-12 medium, fetal bovine serum (FBS), and lipofectamine-based transfection kit were purchased

from Life Technologies, Inc., Grand Island, NY. The affinity-purified, anti-CARP-1 polyclonal antibodies were as described [2]. Antibodies for myc-tag, caspase-9, p38$\alpha/\beta$, and phospho-p38$\alpha/\beta$ were purchased from Cell Signaling, Beverley, MA, while anti-HA tag antibodies were purchased from Covance, Berkeley, CA. The ProBond purification system for affinity purification of TAT-tagged peptides was purchased from InVitrogen, Corp., Carlsbad, CA.

### Recombinant plasmid constructs

The construction of plasmids for expression of myc-His-tagged wild-type CARP-1, as well as mutant CARP-1 proteins, and generation of retroviruses for transduction of CARP-1 proteins has been described before [5]. Vector plasmid pTAT/HA and the plasmid pTAT/HA-eGFP for expression of His-TAT-HA-eGFP have been described elsewhere [10] and were kindly provided by Dr. Steve Dowdy, UCSD, San Diego, CA. Utilizing a combination of PCR and standard cloning methodologies in conjunction with the vector plasmid pTAT/HA, various recombinant plasmids harboring CARP-1 cDNA fragments were generated (depicted in Fig. 5a below). *E. coli* BL21 cells were transformed with each of the recombinant plasmids, eGFP as well as various CARP-1 peptides having HA and poly-histidine tags as well as retroviral TAT transduction domain positioned at the amino termini were affinity purified following our previously described methodology [13].

### Cell lines and cell culture

NIH3T3 derivative PT-67 mouse fibroblasts expressing retroviruses for CARP-1 peptides were cultured and maintained essentially as described [5]. Routine maintenance and culture of NHL cell lines including Raji B-cell line, Jurkat T cells, WSU-DLCL2, and WSU-FSCCL cells was carried out as described previously [14–16]. The WSU-DLCL2 and WSU-FSCCL cells were established from patients with aggressive lymphoma that did not respond well to chemotherapy (including adriamycin) or radiation therapy. WSU-DLCL2 represents a diffuse large cell NHL, grows as subcutaneous (s.c.) tumors remaining near the site of inoculation, and can be established as bilateral tumors in mice where antitumor effect measurements such as T/C, T-C, and Log10kill can be determined. WSU-FSCCL cells represent transformed follicular lymphoma that grows throughout the mouse disseminated from the implantation site (tail vein), homing to bone marrow, spleen, and the bloodstream, where the human graft cells predominate over the host mouse cells by day 14. For example, femur marrow is full of lymphoma cells in the FSCCL model by 14 days. The 22–35 days between graft establishment and the beginning of animal death create an "experimental window" where parameters of drug response, animal health, survival, percent increase in host life span (%ILS) and mechanism of action can be studied. WSU-DLCL2 and WSU-FSCCL xenografts are therefore models for resistant lymphoma.

### Flow cytometric analyses

The flow cytometric evaluation of the cell cycle status and apoptosis was performed as described previously [2]. Briefly, the cells were untreated, transduced with retroviruses encoding wild-type CARP-1, or treated with various TAT-tagged peptides. The cells were collected, washed twice with ice-cold PBS, fixed in 70% ethanol, recentrifuged, washed again with PBS, and stained with 0.5 ml of propidium iodide (0.1% in 0.6% Triton-X in PBS) and 0.5 ml of RNAse A (2 mg/ml) for 45 min in dark. Cells were then sorted on a BD Biosciences FACScan cytometer (San Jose, CA), and the data analyzed using the multicycle program from Phoenix Flow Systems (San Diego, CA).

### Western blot and apoptosis assays

Logarithmically growing cells were either transduced with retroviruses or treated with TAT-tagged peptides for various time periods, and cells were lysed to prepare protein extracts. The protein extracts were electrophoresed on 9–12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with various antibodies to determine expression/presence of the corresponding proteins. In addition, cell lysates were prepared from cells transduced with retroviruses or treated with different affinity-purified peptides and utilized to determine cell growth inhibition and apoptosis levels. The cell viabilities were determined by MTT assays by measuring the absorbance of the converted dye at the indicated wavelength essentially as described [5] or by utilizing LIVE/DEAD viability/cytotoxicity assay (Molecular Probes, Eugene, OR) by following the vendor-suggested protocols. Apoptosis levels were determined by either counting acridine orange-ethidium bromide-stained cells or by utilizing the DNA fragmentation-based ELISA kit (Roche Diagnostics, Indianapolis, IN) as described by us before [5]. In certain instances, caspase-9 activation was measured by utilizing a colorimetric assay kit (Chemicon) to determine apoptosis following previously detailed methods [5]. The apoptosis levels in tumor explants were determined by either immunostaining for activated (phosphorylated) p38 MAPK or by using the terminal deoxynucleotidyl transferase-mediated nick end-labeling assay kit (Roche Diagnostics) as detailed before [13].

### Immunocytochemistry and immunohistochemistry

NHL cells were treated with affinity-purified His-TAT-HA-CARP-1 (1-198) peptide. For immunocytochemical analyses, a 1:2000 dilution of anti-HA-tag monoclonal antibody was used. In addition, arrays of primary DLCL specimens (Table 1 below) were stained. Each specimen was arrayed in triplicate, and expression/levels of several proliferation and/or apoptosis-regulating molecules including CARP-1, $Bcl_6$, activated (phosphorylated) Akt, cleaved caspase-3, IRF-4/MUM-1, and $Bcl_2$ proteins was determined by immunohistochemical methods. The antibody-stained cells were then photographed utilizing Zeiss microscope with attached 35-mm camera for recording the photomicrographs. Staining score was determined independently by two individuals according to the following formula to reflect quantitative as well as qualitative intensity: overall tumor staining was reported as percent staining. The percentage staining was multiplied with the intensity score to obtain a single numerical staining score [Score = Staining intensity (0–3; 0 = negative, 1 = weak, 2 = moderate, and 3 = strong) × % tumor cells staining]. The level of the markers, except cleaved caspase-3, in tumors was considered high when the score was ≥100, while tumors with <100 score were considered low expressers.

### Establishment of tumors in severely compromised immunodeficient (SCID) mice

SCID mice were purchased from Taconic labs (German Town, NY). After a period of adaptation, 2–3 mice were subcutaneously (s.c.) injected on each flank with $\sim 10^6$ WSU-DLCL2 cells. After the tumors developed, the mice were killed, tumors dissected, and small tumor fragments xenografted s.c into fresh group of mice for efficacy studies. Once palpable tumors developed, the mice were randomly grouped ($n = 7$/group) and were treated with affinity-purified proteins at the dose of 25 μg of the respective protein per tumor per day for 5 days. Treatments with adriamycin consisted of intravenous (via tail vein) administration of a dose of 3.3 mg/kg/day for 5 days. The tumors were allowed to grow for another 20 days, and tumor measurements were carried out at multiple time points during the course of treatments and observation periods [14–16]. In addition, $1 \times 10^7$ WSU-FSCCL cells were injected i.p. into the 3-week-old female ICR SCID mice. The animals began to develop lymphoma involving diffuse adenopathy, splenomegaly, infiltration of liver and bone marrow, along with ascites after 2 weeks of transplantation. Six animals per group were either untreated or treated (i.p) with various peptides at a dose of 50 mg/kg/day for five injections. The animals were observed daily, their survival recorded, and euthanized when they appeared ill following previously described procedures [17, 18]. All the efficacy studies were conducted in triplicate under WSU-AIC approved protocol #A09-22-04.

**Table 1** Correlation of CARP-1 expression with cleaved caspase-3, p-Akt, $Bcl_6$, $Bcl_2$, CD10, and IRF-4/MUM1 expression in diffuse large B-cell lymphomas ($n = 83$)

| | | Cleaved caspase-3 | p-Akt | $Bcl_6$ | $Bcl_2$ | CD10 | MUM1 |
|---|---|---|---|---|---|---|---|
| CARP-1 | CC | 0.355 | −0.240 | 0.274 | −0.111 | 0.090 | −0.172 |
| *p* value | 0.001* | 0.029* | 0.012* | 0.323 | 0.417 | 0.119 | |

*CC* Correlation coefficient

* Statistically significant

## Results

### CARP-1 levels correlate with higher apoptotic index in DLBLs

In light of our previous studies indicating an inverse correlation between the CARP-1 levels and the grades of HBC [13], and the fact that ectopic expression of CARP-1 or its peptides inhibited HBC growth in vitro and in vivo [5, 13], we speculated that CARP-1 expression will attenuate growth of NHL cells and its levels might also be of prognostic value for DLBLs. We first investigated potential prognostic value of CARP-1 for DLBLs by utilizing tissue arrays having normal and DLBL samples in conjunction with immuno-histochemical methodologies. In the normal lymphoid tissue (tonsil), CARP-1 expression was predominant in the germinal centers of the reactive follicles that are known to undergo significant apoptosis (Fig. 1). In addition, perinuclear presence of CARP-1 was also noted in a representative tissue specimen from DLBL (Fig. 1). Next, expression/levels of several proliferation and/or apoptosis-regulating molecules including CARP-1, $Bcl_6$, activated (phosphorylated) Akt, Nur77, cleaved caspase 3, IRF-4/MUM-1, and $Bcl_2$ proteins in the DLBL tumor arrays was determined as in "Materials and methods". The immuno-histochemical analyses revealed CARP-1 expression in 66/83 (80%) of the diffuse large cell lymphomas (Table 1). Of these cases, 16/83 (19%) had high CARP-1 expression, while 50/83 (60%) had low levels of CARP-1. When the cases were grouped as low apoptotic index versus high apoptotic index, based on a cut-off value of 10 apoptotic cells per high power field and CARP-1 expression, there was a significant association of apoptosis and high CARP-1 expression ($p = 0.001$). Further CARP-1 expression also correlated with levels of $Bcl_6$ ($p = 0.012$). On the other hand, levels of activated Akt correlated inversely ($p = 0.029$) with CARP-1 presence. Interestingly,

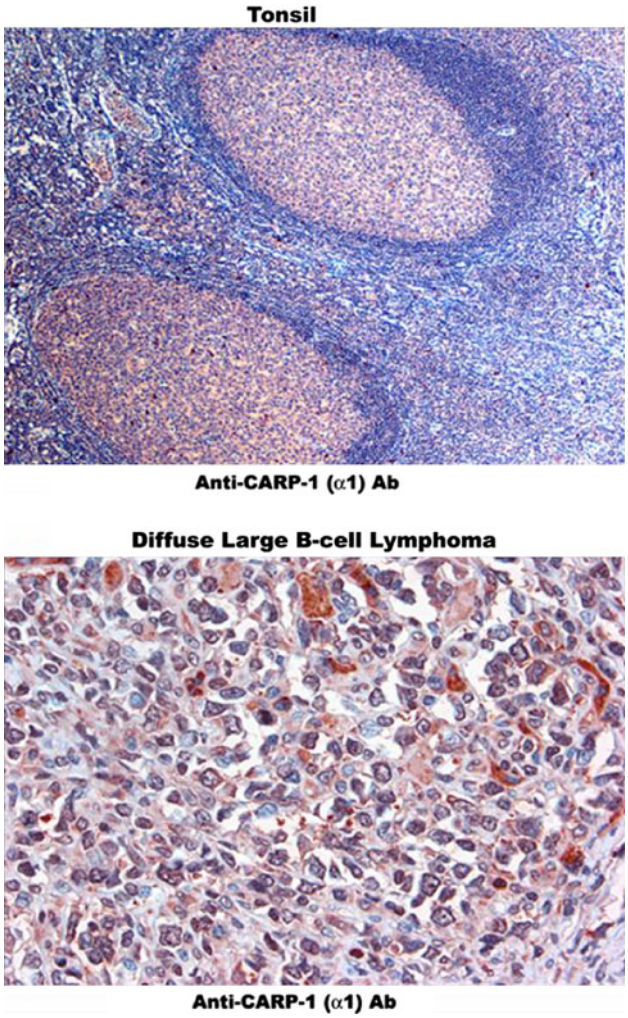



**Fig. 1** CARP-1 expression in a normal tonsil tissue (upper; ×40 magnification) and a representative case of DLBL (lower; ×200 magnification) following immunohistochemical staining using anti-CARP-1 (α1) antibody. CARP-1 presence is indicated by *dark brown* staining in both the photomicrographs

although not statistically significant, an inverse correlation was also apparent for expression of IRF-4/MUM-1 or $Bcl_2$ proteins when compared with the levels of CARP-1 in DLBLs (Table 1). Overall, in light of strong staining for CARP-1 in the reactive germinal centers, our observations are suggestive of an association of CARP-1 expression with a germinal center phenotype in the DLBLs examined.

## Adriamycin induces CARP-1 expression in lymphoma cells

The anthracycline class of agents inhibits growth of cancer cells in part by inducing apoptosis and are the choice of therapies that are currently utilized against a variety of neoplastic conditions including lymphomas. Since CARP-1 has previously been found to regulate apoptosis by adriamycin and etoposide in HBC cells [2, 3], we investigated whether adriamycin induces CARP-1 in NHL cells. The Jurkat T cell, Raji Burkitt lymphoma, WSU-DLCL2, and WSU-FSCCL lymphoma cells were separately treated with adriamycin (20 ng/ml, equivalent to ~34 nM), followed by immunocytochemical and Western immunoblot analyses of untreated and adriamycin-treated cells utilizing anti-CARP-1 (α2) antibodies to determine CARP-1 levels. CARP-1 expression was elevated in the adriamycin-treated Raji Burkitt lymphoma and WSU-FSCCL (Fig. 2a). Although Jurkat and WSU-DLCL2 cells expressed CARP-1, adriamycin failed to elevate CARP-1 expression in these cells (Fig. 2a). Immunocytochemical staining of adriamycin-treated and untreated NHL cells corroborated stimulation of CARP-1 expression in adriamycin-treated Raji Burkitt lymphoma and WSU-FSCCL cells (Fig. 2b).

## Expression of CARP-1 or its mutants suppresses growth of NHL cells

Since adriamycin treatments stimulate CARP-1 in certain NHL cells, we first determined whether adriamycin exposure induced apoptosis in NHL cells where it failed to induce CARP-1 expression. To test this possibility, we treated Jurkat cells with 20 ng/ml adriamycin for 24 h and determined their cell cycle distribution by flow cytometric analysis following their labeling with propidium iodide. As shown in Fig. 3, the untreated control cells had 3% cells in sub-G0, 47% in G1, 22% in S and 28% in G2M phases of cell cycle. The treatment with adriamycin elicited minimal to absent apoptosis and consequent alterations in their cell cycle distribution with 3% cells in sub-G0, 48% in G1, 27% in S, and 22% in G2M phases. Consistent with the flow cytometric analyses and data in Fig. 2, treatments with adriamycin failed to induce CARP-1 expression in these cells. We next examined whether CARP-1 expression will promote apoptosis. Jurkat cells were transduced with ~$2 \times 10^4$ cfus of retroviruses encoding CARP-1 [2] for 24 h as detailed in "Materials and methods". Flow cytometric analysis of these cells revealed 3% of cells in sub-G0, 47% in G1, 24% in S, and 26% in G2M in control untreated cells, while CARP-1-transduced cells elicited a 56% sub-G0 fraction, 31% G1 fraction, 15% S fraction, and 8% G2M fraction. Thus, CARP-1 expression resulted in a significant increase (from 3 to 56%) in the apoptotic fraction when compared with their untreated (control) counterparts (Fig. 3). Since adriamycin treatment failed to induce CARP-1 expression in Jurkat and WSU-DLCL2 cells, while over-expression of CARP-1 stimulated apoptosis in Jurkat cells, we next examined whether ectopic expression of CARP-1 or its mutant proteins will also inhibit growth of WSU-DLCL2 cells. Here, the cells were either untreated (control), treated with 20 ng/ml adriamycin

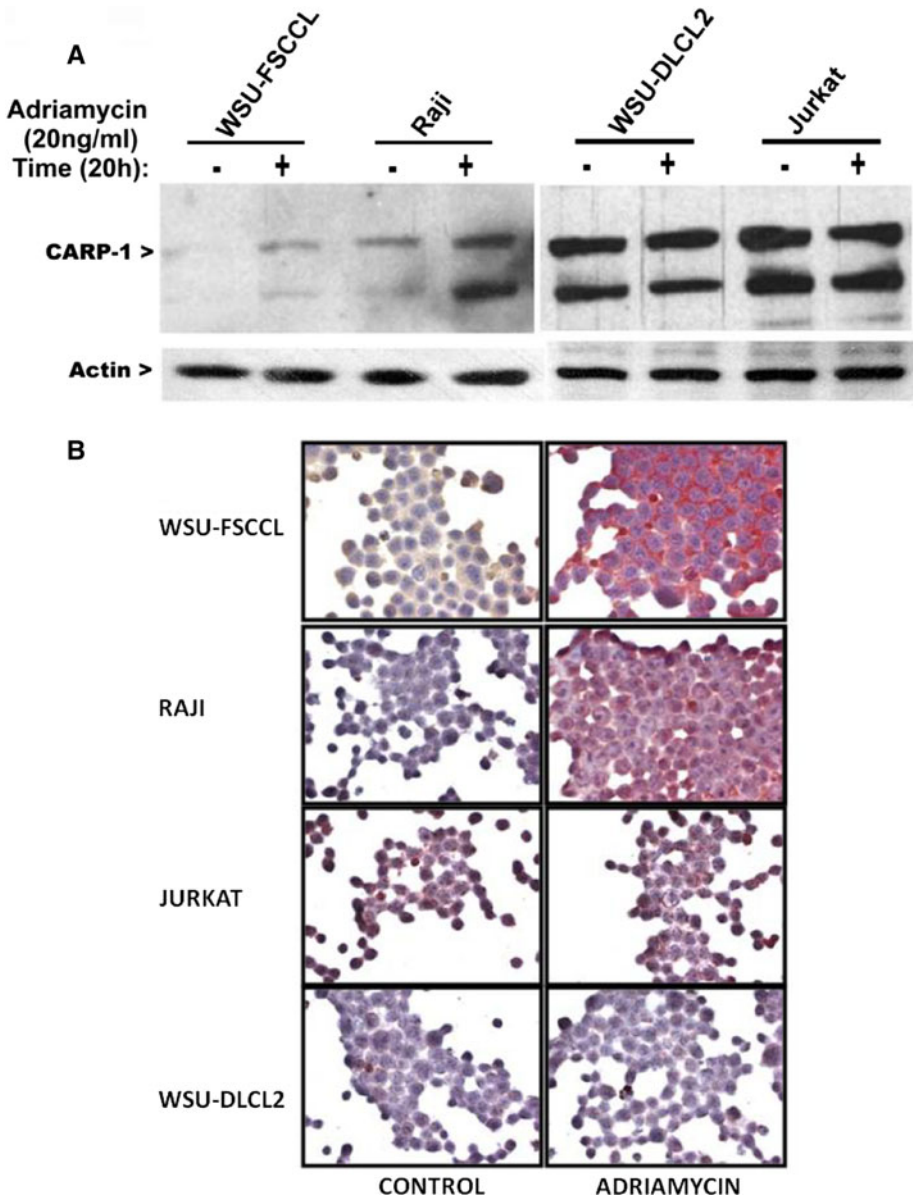



**Fig. 2** Adriamycin-dependent increase in CARP-1 expression in NHL cells. **a** The cells were either untreated (indicated as −) or treated with adriamycin (indicated as +) as noted. At the end of the treatment period, the cells were lysed, and 100 micrograms of respective protein lysates from each sample were analyzed on SDS–PAGE followed by immunoblotting with anti-CARP-1 (α2) antibodies as detailed in "Materials and methods". As a control for sample loading, the membrane was re-probed with anti-actin antibodies. The presence of CARP-1 and actin proteins is indicated on the left side of respective blots. **b** The NHL cells were either untreated (indicated as CONTROL) or treated with 20 ng/ml of adriamycin for 20 h. The cells were then fixed and stained with anti-CARP-1 (α2) antibodies following immunocytochemical procedures detailed in "Materials and methods". Elevated levels of CARP-1 are notable by the *dark brown* staining in the photomicrographs of adriamycin-treated FSCCL and Raji cells

for 24 h, or transduced with retroviruses encoding wild-type or mutant CARP-1 proteins. As shown in Fig. 4a, expression of wild-type or mutant CARP-1 proteins attenuated growth of the WSU-DLCL2 cells when compared with their untreated, adriamycin-treated or vector-transduced counterparts. Although expression of CARP-1 mutants 452-654, 197-454, and 603-898 caused modest growth suppression that was not statistically significant, expression of wild-type CARP-1 or its mutants 1-198 and 896-1150 nonetheless caused significant loss of viabilities of these cells. Moreover, cell growth inhibition by CARP-1 (1-198) was not significantly different to that noted for the cells that were transduced with wild-type CARP-1 or CARP-1 (896-1150) proteins. Our previous studies have shown that the amino-terminal 1-198 region of CARP-1 was a target of apoptosis signaling following EGFR blockage, and expression of CARP-1 (1-198) protein inhibited growth of HBC cells in vitro and in vivo in part by promoting apoptosis [5, 13]. We then examined whether and to what extent expression of CARP-1 (1-198) alone or in combination with adriamycin inhibited growth of NHL cells. WSU-DLCL2 and Raji cells were either untreated (control), treated with 20 ng/ml adriamycin for 24 h, transduced with retroviruses encoding CARP-1 (1-198) protein, or subjected to a combination of adriamycin and retroviruses for CARP-1 (1-198) protein. Following the conclusion of the treatment periods, the cell viabilities were measured as in "Materials and methods". As shown in Fig. 4b, transduction of both the cells with vector-encoding viruses did not affect their viabilities when compared with their untreated counterparts. Treatment with adriamycin on the other hand inhibited growth of the Raji

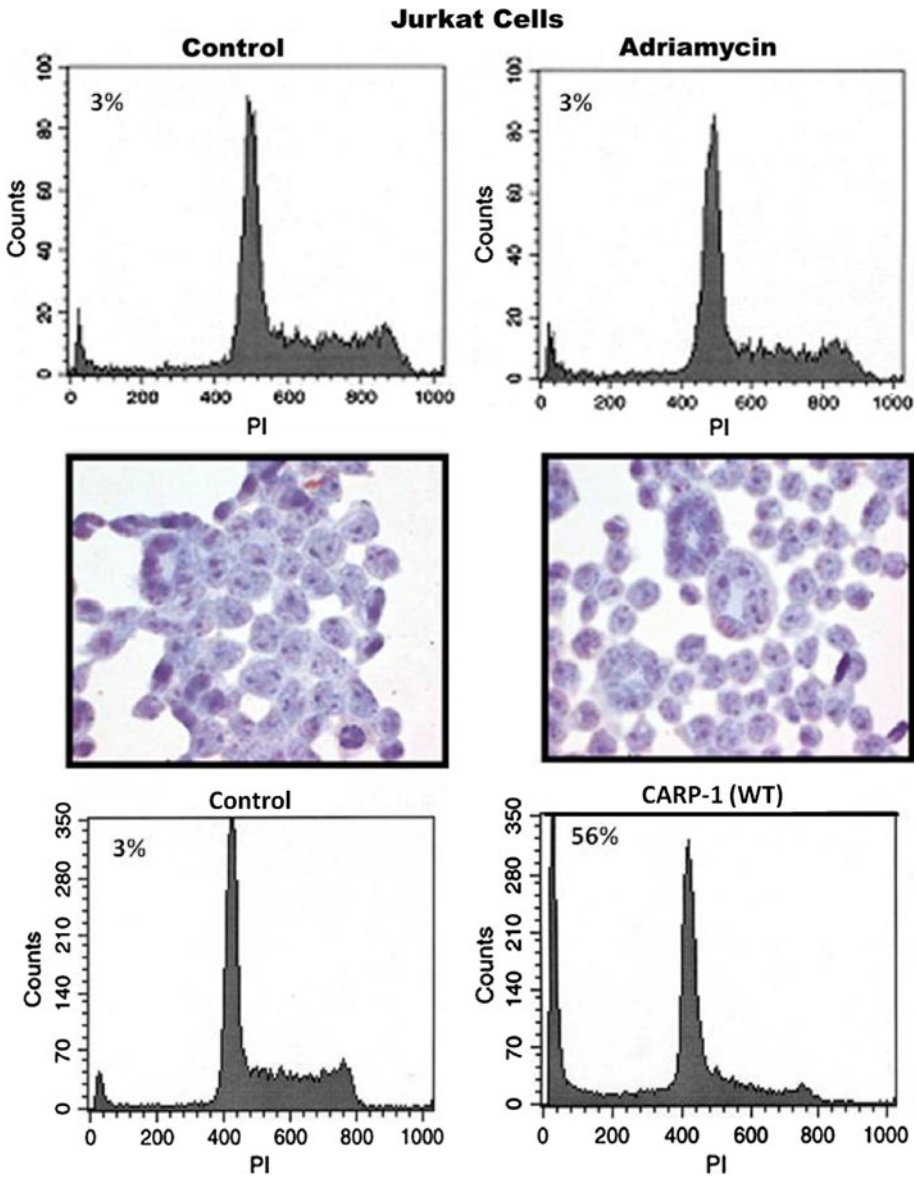
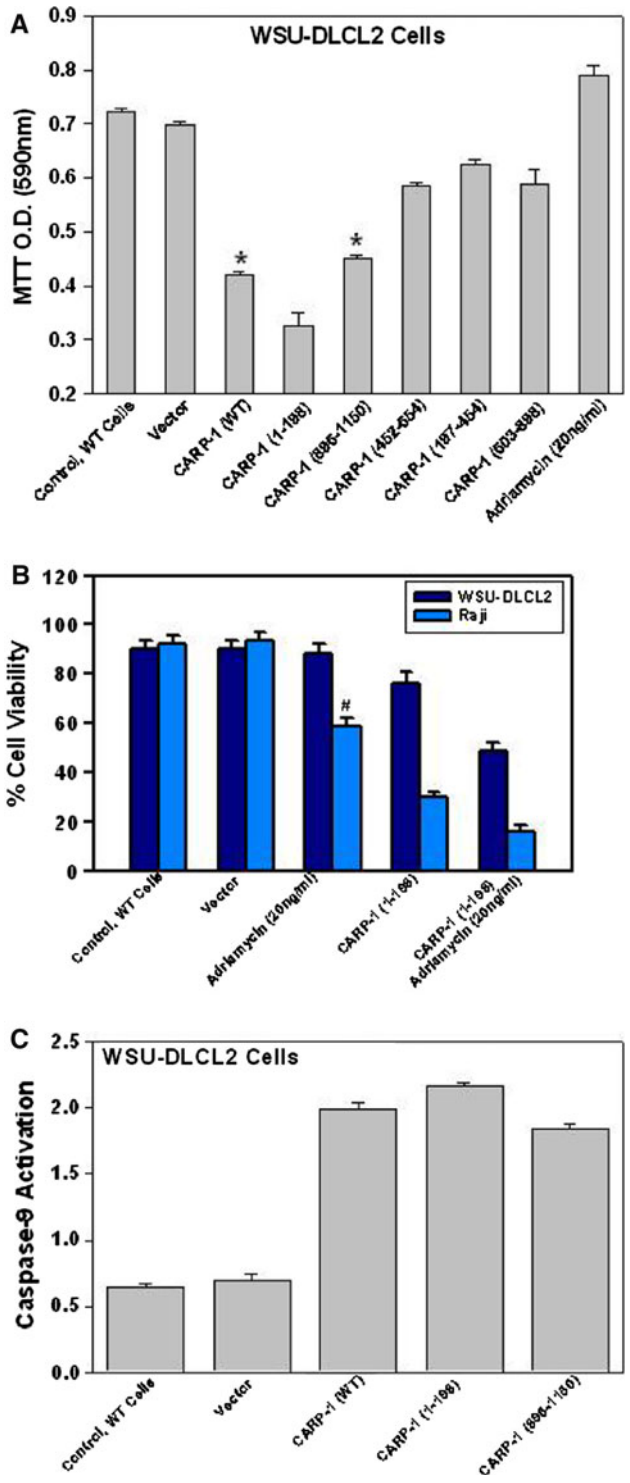



**Fig. 3** CARP-1 expression induces apoptosis. Jurkat cells were either untreated (control) or treated with 20 ng/ml dose of adriamycin for 20 h. Cells were either labeled with propidium iodide followed by their sorting by flow cytometry (*top left* and *right*) or fixed and labeled with anti-CARP-1 (α2) antibodies as in Fig. 2 (*middle left* and *right*). Jurkat cells were also transduced with retroviruses encoding wild-type CARP-1 as described in "Materials and methods". The untransduced (control) or the CARP-1-transduced cells were evaluated for apoptosis by flow cytometric analyses as above. Apoptotic rate of the untreated, adriamycin-treated, or CARP-1-transduced cells was determined by calculation of the sub-$G_0$ fraction (indicated as % numbers)

cells, while failed to induce significant loss of viabilities of WSU-DLCL2 cells. Although exposure to retroviruses encoding CARP-1 (1-198) for 48 h inhibited growth of WSU-DLCL2 cells (Fig. 4a), retroviral expression of CARP-1 (1-198) for a period of 16 h failed to significantly inhibit their growth (Fig. 4b). Combined presence of CARP-1 (1-198) and adriamycin however inhibited growth of both the cell types to a greater extent when compared with their inhibition in the presence of CARP-1 (1-198). These data therefore suggest that CARP-1 expression likely sensitizes NHL cells to growth inhibition by adriamycin. Whether CARP-1 or its mutant proteins suppressed growth of NHL cells by inducing apoptosis was investigated in WSU-DLCL2 cells. Cells were either untransduced or transduced with viruses encoding vector, CARP-1 wild-type or its mutant 1-198 and 896-1150 proteins. Consistent with our observations in the breast cancer model [5], ectopic expression of wild-type CARP-1 or its mutants stimulated activation of caspase-9 in WSU-DLCL2 cells (Fig. 4c). These data collectively suggest that expression of CARP-1 or its mutant proteins, specifically CARP-1 (1-198) or CARP-1 (896-1150), inhibit growth of WSU-DLCL2 cells in part by stimulating apoptosis, and expression of the apoptosis-inducing CARP-1 (1-198) mutant enhances efficacy of adriamycin to suppress growth of the NHL cells. Since exposure to adriamycin resulted in elevated CARP-1 in Raji cells but not in WSU-DLCL2 cells, combined presence of adriamycin and CARP-1 (1-198) was more effective in suppressing growth of both the Raji and WSU-DLCL2 NHL cells when compared with the either agent alone indicate that adriamycin likely inhibits NHL cell growth in part by elevating CARP-1 levels.




◀ **Fig. 4** Expression CARP-1 or its mutants inhibits NHL cell growth. **a**, **b** Indicated cells were either untreated or treated with noted retroviruses, adriamycin or a combination of both. All the cells were treated with 5 ml of respective viral supernatants for 48 h in **a**, while for **b** the retroviral, adriamycin, or combination treatments were for a period of 16 h. The cell viabilities were determined by MTT assays by measuring the absorbance of the converted dye at the indicated wavelength essentially as described [5]. Columns, means of three or more independent experiments; bars, SE. * and # denote $p < 0.01$ relative to untreated, control WT cells. **c** Cells were either untreated (control, WT cells) or treated with noted retroviruses as in A. Caspase-9 activation was determined by utilizing a colorimetric assay kit (Chemicon). Columns represent means of three independent experiments; bars, SE

## TAT-tagged CARP-1 peptides inhibit NHL growth in vitro and in vivo

In light of our earlier studies demonstrating presence of multiple, apoptosis-promoting subdomains within CARP-1 protein [5] and data in this study indicating NHL growth inhibition by retroviruses encoding wild-type or mutant CARP-1 proteins, we speculated that affinity-purified CARP-1 peptides will inhibit growth of NHL cells and may have potential utility as anti-NHL agents. As a first step to test this possibility, we subcloned CARP-1 cDNA fragments into vector plasmid pTAT/HA to generate His-TAT-HA-CARP-1 peptides. Figure 5a shows a schematic of various recombinant plasmids. The TAT tag belongs to a class of peptide molecular transporter domains (also known as protein transduction domains, PTDs). PTDs are 10–30 amino acid residues in length and are rich in basic amino acids, e.g., arginine and lysine. Transduction of proteins bearing PTDs does not depend on classical receptor-mediated, transmembrane, or endocytic pathways and is a novel and emerging strategy to transport proteins across the plasma membrane of eukaryotic cells [10, 13]. The recombinant plasmids were generated for expression of His-TAT-HA-CARP-1 (1-198), (197-454), (452-654), or (896-1150) peptides. His-TAT-HA-tagged CARP-1 peptides and eGFP protein were affinity-purified from *E. coli* (BL-21) lysates, and their purity validated by SDS–PAGE analyses following our recently described protocols [13]. As shown in Fig. 5b, affinity purification procedures yielded a single peptide of the expected size for all but His-TAT-HA-CARP-1 (197-454) proteins. The preparation of His-TAT-HA-CARP-1 (197-454) protein however contained multiple, smaller-sized peptides along with the expected ~35-kD parent peptide. Western immunoblotting of the membrane containing these affinity-purified proteins with anti-HA-tag antibodies further validated the smaller-sized peptides in His-TAT-HA-CARP-1 (197-454) protein preparation (not shown). Whether the smaller-sized peptides in His-TAT-HA-CARP-1 (197-454) preparations arise from the carboxyl-terminus truncations of the CARP-1 (197-454) peptide and the extent these truncated peptides interfere with the NHL cell growth remains to be clarified. The WSU-DLCL$_2$ cells were incubated with 50 μg/ml of affinity-purified His-TAT-HA-CARP-1 (1-198). Immunocytochemical staining of cells with anti-His-tag antibodies revealed the presence of His-TAT-HA-CARP-1 (1-198) in the cytoplasmic compartment of the NHL cells (not shown)

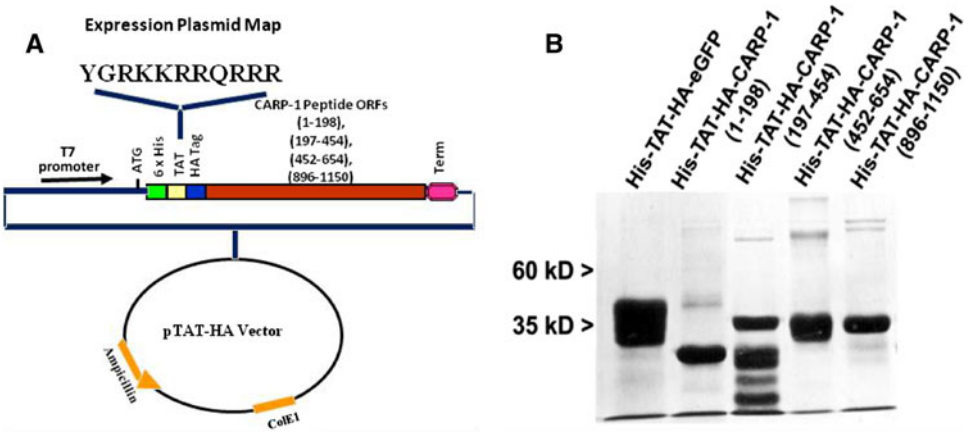



**Fig. 5** Generation and affinity purification of TAT-tagged CARP-1 peptides. **a** Schematic diagram of the pTAT-HA vector plasmid with location of various epitope tags fused to CARP-1 peptide open reading frames (ORFs). **b** The recombinant plasmids were propagated into *E. coli* and tagged peptides were affinity purified as in "Materials and methods". The photograph shows coomassie-stained SDS–PAGE having indicated affinity-purified peptides. Approximate locations of two molecular weight standard markers are indicated on the *left*

further corroborating our earlier observations of transport of the TAT-tagged CARP-1 peptides across the plasma membranes of HBC cells [13]. We have also previously observed that the TAT-tagged CARP-1 peptides, with the exception of TAT-eGFP protein, suppressed growth of HBC cells in vitro as well as in vivo, and this effect was due, in part, to stimulation of apoptosis [13]. We then examined whether TAT-tagged CARP-1 peptides will also suppress NHL cell growth. WSU-DLCL$_2$ cells were incubated with His-TAT-HA-CARP-1 (1-198) or His-TAT-HA-eGFP proteins alone or in combination with adriamycin, and number of live, viable cells determined as in "Materials and methods". His-TAT-HA-CARP-1 (1-198) significantly inhibited NHL cell growth, and in combination with adriamycin caused an almost complete loss of viability of these cells (Fig. 6a). Moreover, the loss of viabilities of NHL cells in the presence of CARP-1 peptides, adriamycin, or a combination of both of the agents was due in part to increased apoptosis (Fig. 6b). We further investigated the mechanisms of NHL growth suppression by treating the WSU-FSCCL cells, in vitro, with different TAT-tagged peptides or adriamycin, followed by flow cytometric analyses. Adriamycin treatment caused $G_2M$ arrest as well as apoptosis in these cells (Fig. 6c). Treatments with TAT-tagged peptides also resulted in significant apoptosis when compared with eGFP-treated cells. Interestingly, presence of TAT-CARP-1 peptides elicited higher accumulation of these cells in G1 and apoptotic fractions when compared with their adriamycin-treated counterparts. Data in Fig. 6c suggest that in certain NHL types such as FSCCL, adraimycin, and CARP-1 peptides elicit arrest/accumulation of cells in distinct phases of cell cycle, and thus a combination therapy of TAT-tagged CARP-1 peptides and adriamycin will likely be more efficacious in suppressing growth of these lymphomas.

We recently demonstrated that intra-tumoral administration of TAT-CARP-1 (1-198) peptide, but not its variant with Y192/F substitution, inhibited growth of HBC cell-derived tumor xenografts [13]. Whether TAT-tagged CARP-1 (1-198) peptide will attenuate growth of WSU-DLCL$_2$ cell-derived xenografts was investigated next. WSU-DLCL2 cell-derived tumor xenografts were established following our published protocols [18]. The tumors were either untreated (control) or administered with His-TAT-eGFP, His-TAT-HA-CARP-1 (1-198), His-TAT-HA-CARP-1 (896-1150), or adriamycin as in "Materials and methods". Administration of His-TAT-HA-CARP-1 (1-198) or adriamycin caused significant ($\sim$40%) reduction in tumor burden when compared with the tumor weights of control animals (Fig. 7a). Moreover, administration of His-TAT-HA-CARP-1 (896-1150) also attenuated tumor growth when compared with His-TAT-HA-eGFP-treated or untreated (control) counterparts (Fig. 7b). Immuno-histochemical analyses of the xenografted tumor biopsies from the treated animals showed elevated levels of phosphorylated p38 MAPK as well as apoptosis (Fig. 7d), suggesting that TAT-tagged CARP-1 (1-198) and (896-1150) peptides inhibit lymphoma xenograft growth in part by stimulating apoptosis. These observations are also consistent with our data from HBC cell-derived tumor xenograft model [13].

In a preliminary study, various doses of His-TAT-HA-CARP-1 (1-198) were injected in the SCID mice by tail vein to determine maximal tolerated dose (MTD). The mice were injected with 5, 50, and 100 mg/kg/day for 5, 3, and 3 consecutive injections, respectively. The mice were observed for subsequent 3 weeks and did not show any visible toxicity including symptoms of diarrhea, dehydration, weight loss, hair loss, or any other discomfort. The 100 mg/kg/day for 3 consecutive injections caused acute toxicity. In a related experiment, SCID mice having

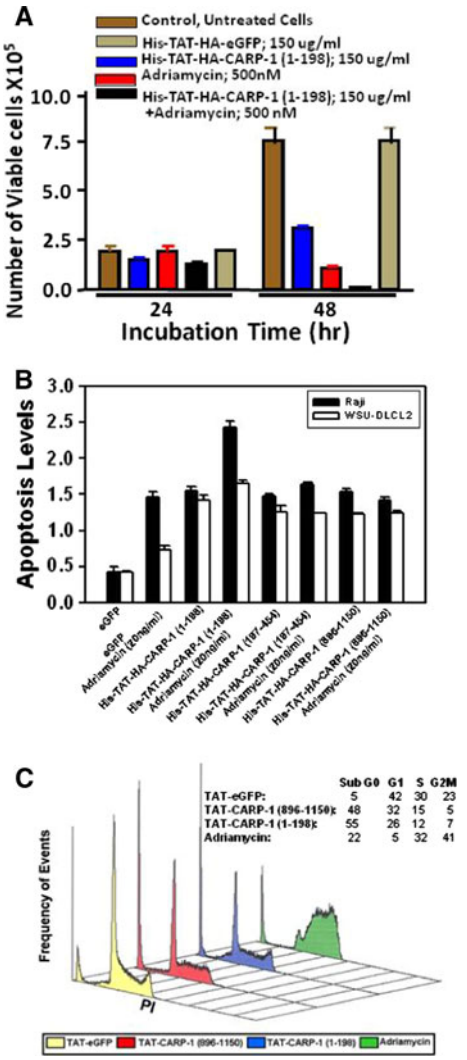



**Fig. 6** TAT-tagged CARP-1 peptides inhibit NHL cell growth and induce apoptosis. Cells were either incubated with indicated dose of adriamycin for 24 h, peptide alone for 48 h, or preincubated with peptide for 24 h followed by treatment with adriamycin (20 ng/ml) for further 24 h. In **a**, number of viable *WSU-DLCL*$_2$ cells was determined by utilizing LIVE/DEAD viability/cytotoxicity assay (Molecular Probes, Eugene, OR), while in **b**, apoptosis was measured by counting acridine orange-ethidium bromide–stained cells as described by us before [13]. The columns represent means of three independent experiments; bars, SE. In **c**, cells were labeled with propidium iodide following their treatments with indicated agents. Fractionation of the labeled cells was carried out by flow cytometry as in "Materials and methods"

SKBR-3 HBC cell-derived tumor xenografts were administered TAT-tagged eGFP or TAT-CARP-1 (1-198) at a dose of 14 or 17 mg/kg/day, respectively, for 10 days by s.c. route (behind the neck, away from the tumor site). Immuno-histological analyses revealed presence of eGFP as well as CARP-1 (1-198) proteins in the tumors (not shown), indicating translocation of these proteins to the tumor sites. These observations corroborate previous report where TAT-tagged biologically active peptide was delivered to various mouse tissues following intraperitoneal (i.p.) injections [19].

In an analogous approach, $1 \times 10^7$ WSU-FSCCL cells were injected i.p. into the 3-week-old female ICR SCID mice. The animals began to develop lymphoma involving diffuse adenopathy, splenomegaly, infiltration of liver and bone marrow, along with ascites after 2 weeks of transplantation. The mice were treated by i.p. injections of TAT-tagged peptides, observed daily and euthanized when they appeared ill as described [17, 20]. Administration of TAT-tagged CARP-1 (1-198) significantly enhanced survival of animals bearing WSU-FSCCL-derived lymphomas when compared with TAT-tagged eGFP-treated or untreated animals (Fig. 7c). These observations collectively suggest that the TAT-tagged peptides are likely well tolerated and bio-available to lymphoma cell-derived tumors when administered to animals by various routes. Together, data in Fig. 7 also demonstrate that TAT-tagged CARP-1 peptides inhibit NHL growth in vivo in part by activating apoptosis signaling.

## Discussion

CARP-1 is a co-activator of steroid-thyroid superfamily of transcription factors as well as the tumor suppressor p53 [3] and functions as a key regulator of apoptosis signaling by a variety of agents including chemotherapeutic adriamycin and the EGFR inhibitor Iressa [2, 5]. The lymphoid malignancies such as DLBLs are frequently treated with regimens that include adriamycin. The mechanism of action of adriamycin involves pleiotropic effects including (a) activation of signal transduction pathways, (b) generation of reactive oxygen species (ROS), (c) stimulation of apoptosis, and (d) inhibition of DNA topoisomerase II catalytic activity (comprehensively reviewed in Ref. [21]). In addition, two major endogeneous modulators of apoptosis, Bcl-2 and p53 tumor suppressor protein, as well as their associated downstream effectors, are known to regulate adriamycin-dependent growth inhibition [22]. Nevertheless, the order of adriamycin-dependent growth inhibitory signals at the molecular levels remains to be fully defined. Since the drug-resistant lymphomas are encountered in the clinic with increasing frequency, we undertook this investigation to determine whether lymphoma growth inhibition by adriamycin involves CARP-1. If so, should the ectopic expression of CARP-1 inhibit the resistant lymphomas, it will provide a proof-of-principle approach with potential application in targeting resistant lymphomas. To test our hypothesis, we utilized Raji B-cell line, Jurkat T cells, WSU-DLCL2, and WSU-FSCCL lymphoma cells to investigate involvement of CARP-1 in

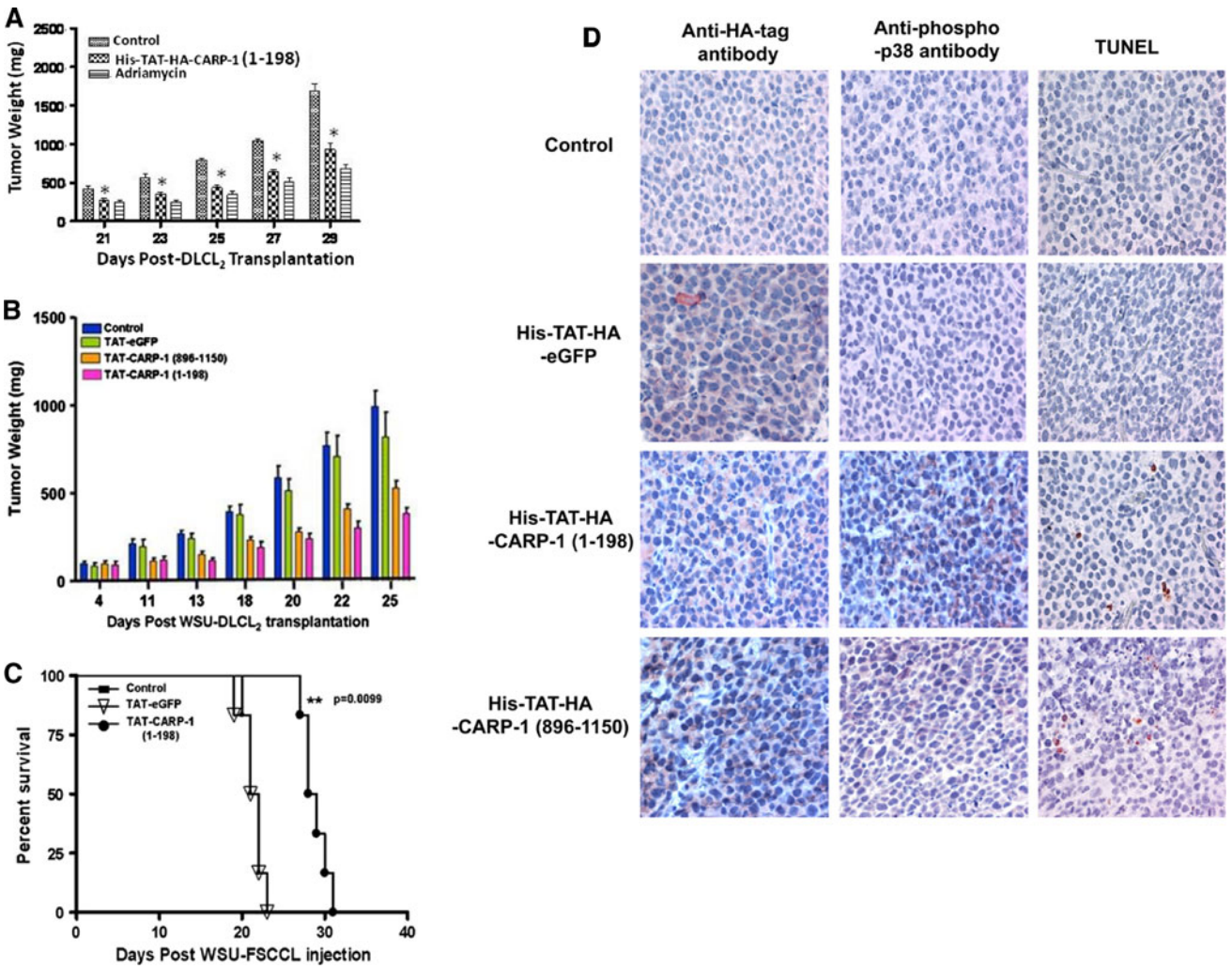



**Fig. 7** TAT-tagged CARP-1 peptides inhibit growth of NHL cell-derived tumor xenografts in SCID mice. The efficacy studies were carried out as in "Materials and methods", and data were analyzed essentially as described before [13]. **a** and **b** Histograms show tumor weight (in mg; mean ± SD). * represent $p$ values of 0.0015, compared to the corresponding His-TAT-HA-eGFP-treated tumors. In **c**, histogram indicates survival of mice (in days) bearing WSU-FSCCL cell-derived disseminated lymphomas. Animals were either untreated or treated with noted peptides as in "Materials and methods". In **d**, the formalin-fixed tumor xenograft biopsies from **b** were paraffin embedded, processed, and subsequently subjected to immunohistochemical staining as detailed in "Materials and methods". Representative photomicrograph (×200 magnification) showing intratumoral presence (brown coloration) of respective peptides is indicated by staining with anti-HA-Tag antibodies (*left column*). Representative photomicrographs (×200 magnification) are also presented demonstrating apoptosis in xenografted tumors following their staining with anti-phospho-p38 antibodies (*middle column*), or using TUNEL assay (*right column*)

regulating their growth in the presence of adriamycin. Of note is the fact that the WSU-DLCL2 and WSU-FSCCL represent models of resistant lymphoma since these cells were established from patients with aggressive lymphoma that did not respond well to chemotherapy (including adriamycin) or radiation therapy, and both of these cells grow well as xenografts in immune-compromised animals. The flow cytometric analyses coupled with MTT assays in Figs. 3 and 4 revealed that Raji cells were sensitive to inhibition by lower doses of adriamycin, while the Jurkat and WSU-DLCL2 cells appeared resistant to adriamycin effects. Western immunoblotting as well as immunostaining of the adriamycin-treated and untreated NHL cells indicated presence of CARP-1 in all these cell types. CARP-1 levels however were elevated in adriamycin-treated Raji and WSU-FSCCL cells but not in adriamycin-treated WSU-DLCL2 or Jurkat cells. Since adriamycin induced CARP-1 expression and suppressed growth of Raji cells, while it did not stimulate CARP-1 in WSU-DLCL2 and Jurkat cells that were refractory to inhibition by low doses of adriamycin suggests that induction of CARP-1 may be a determinant in transducing inhibitory effects of adriamycin. Together with previous studies demonstrating abrogation of apoptotic effects of

adriamycin following knock-down of CARP-1 in HBC cells [2, 3] and our data in Fig. 3 demonstrating apoptosis in CARP-1-transduced Jurkat cells suggest that defects in signaling upstream of CARP-1 likely contribute to lack of adriamycin sensitivity of WSU-DLCL2 and Jurkat cells. Since CARP-1 harbors multiple, apoptosis-inducing epitopes including the N-terminal 1-198 region that is a target of EGFR signaling [5], elucidation of the upstream signal(s) that regulate CARP-1 expression and their role(s) in transducing inhibitory effects of adriamycin in NHL cells could potentially allow for development of strategies for effective utilization of adriamycin in management of resistant lymphomas.

A combination of adriamycin and N-terminal region CARP-1 mutant [CARP-1 (1-198)] elicits greater lymphoma cell growth suppression and apoptosis when compared with either agent alone (Figs. 4b, 6a, b). Moreover, the fact that TAT-tagged CARP-1 (1-198) is also effective in suppressing growth of xenografted resistant NHL in mice (Fig. 7a, b) would indicate for potential utility of this agent in enhancing adriamycin efficacy in NHL. In addition, since expression of CARP-1 (1-198) protein having substitution of tyrosine198 to phenylalanine interferes with HBC cell growth inhibition by EGFR-targeted agent iressa [5], and the TAT-tagged CARP-1 (1-198) was effective in suppressing growth of HBC cell-derived xenografts [13], the NHL growth inhibitory properties of this peptide reagent having N-terminal 198 amino acids of CARP-1 (Fig. 7) underscores its potential for anti-cancer use in a range of malignancies including the ones that are dependent on the cell surface EGFR signaling. Our in vivo data in Fig. 7 also demonstrates anti-NHL properties of a different CARP-1 peptide that has the carboxyl terminal region of CARP-1 [CARP-1 (896-1150)]. Although previous mutagenesis studies suggested for the presence of multiple, non-overlapping apoptosis-promoting subdomains within the CARP-1 protein [5], the fact that TAT-tagged CARP-1 (896-1150) was also effective in suppressing growth of xenografted NHL cells, further highlights the existence of distinct CARP-1 epitopes for transducing growth inhibitory signals in vitro and in vivo. The extent these distinct CARP-1 epitopes are targeted by specific or overlapping upstream signals to elicit cell growth suppression is yet to be clarified. Nonetheless, expression of either the 1-198 or 896-1150 peptides of CARP-1 inhibits cell growth by inducing apoptosis and, in this context, support an apoptosis-promoting property of CARP-1.

In recent years, several studies were conducted to investigate the molecular basis of heterogeneity in DLBLs. These studies revealed the existence of at least two broad subtypes of DLBLs, namely germinal center type and the activated cell type. A third category also exists, which shows an intermediate phenotype. It is widely accepted that the germinal center phenotype is associated with a better prognosis and better response to chemotherapy. The molecular analyses of the clinical heterogeneity among DLBLs have largely focused on individual candidate genes with known functions in other malignancies or in normal lymphocyte development. A growing list of such candidate genes includes adhesion molecules that regulate trafficking of B cells and tumor cells, apoptosis regulatory proteins, and angiogenic peptides that regulate development of an effective tumor vasculature. Most notably, expression of vascular endothelial growth factor (VEGF) is elevated in fatal/refractory DLBLs and associates with adverse outcome, while transcription factor E2F is over expressed in cured DLBLs and is often associated with favorable outcome [23, 24]. In addition, recent gene array-based study revealed that expression of NOR1, a member of the nuclear orphan steroid receptor family that includes Nur77, was associated with favorable outcome in DLBLs [8]. Our immunohistochemical data utilizing DLBL tumor arrays revealed an association of CARP-1 expression with the germinal center phenotype subtype of the DLBLs, which have better prognoses than the activated B-cell phenotype DLBLs and thus argues for an important role for CARP-1 in the biology of DLBLs.

The precise mechanism(s) by which CARP-1 or its 1-198 and 896-1150 peptides induce apoptosis are subjects of our ongoing studies. Although CARP-1 (1-198) is a target of phosphorylation by upstream signaling by EGFRs [5], the proteomic-based studies have revealed multiple serine, threonine as well as tyrosine phosphorylation sites within the wild-type CARP-1 [25–27]. Since the TAT-tagged CARP-1 (1-198) peptide having substitution of tyrosine192 to phenylalanine fails to suppress growth of the HBC cell-derived xenografts in mice [13], whether adriamycin-dependent NHL growth inhibition signaling involves CARP-1 phosphorylation outside and within the 1-198 and 896-1150 regions of CARP-1, and the extent the phosphorylation regulates apoptotic functions of CARP-1 and the identity of the upstream kinases remains to be elucidated.

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