Combination with liposome-entrapped, ends-modified *raf* antisense oligonucleotide (LErafAON) improves the anti-tumor efficacies of cisplatin, epirubicin, mitoxantrone, docetaxel and gemcitabine

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Raf-1 protein serine/threonine kinase plays an important role in cell proliferation and cell survival. We have previously described a novel cationic liposome-entrapped formulation of *raf* antisense oligodeoxyribonucleotide (LErafAON) and its use as a radiosensitizer. The aim of this study was to examine the effect of combination of LErafAON and a chemotherapeutic agent on growth of human prostate (PC-3) and pancreatic tumor xenografts in athymic mice (Aspc-1 and Colo 357). In PC-3 tumor-bearing mice, administration of a combination of LErafAON (i.v., 25 mg/kg/dose, ×10/16) and cisplatin (i.v., 11.0 mg/kg/dose, ×3), epirubicin (EPI) (i.v., 9.0 mg/kg/dose, ×3) or mitoxantrone (MTO) (i.v., 2.5 mg/kg/dose, ×3) led to enhanced tumor growth inhibition as compared with single agents (LErafAON + cisplatin versus cisplatin, $p<0.0002$, $n=8$; LErafAON + EPI versus EPI, $p<0.0001$, $n=6$; LErafAON + MTO versus MTO, $p<0.05$, $n=5$). In prostate or pancreatic tumor-bearing mice, combination of LErafAON (i.v., 25 mg/kg/dose, ×10/13) with docetaxel (Taxotere) (i.v., 5, 7.5 or 10 mg/kg/dose, ×2/4) led to tumor regression or enhanced growth inhibition as compared with single agents (PC-3: LErafAON + Taxotere versus Taxotere, $p<0.02$, $n=7$; Aspc-1: LErafAON + Taxotere versus Taxotere, $p<0.03$, $n=5$; Colo 357: LErafAON + Taxotere versus Taxotere, $p<0.04$, $n=7$). Combination of LErafAON (i.v., 25 mg/kg/dose, ×10/13) with gemcitabine (i.v., 75 mg/kg/dose, ×4/6) also caused a significant tumor growth inhibition in the two pancreatic carcinoma models studied (Aspc-1: LErafAON + gemcitabine versus gemcitabine, $p<0.0001$, $n=7$; Colo 357: LErafAON + gemcitabine versus gemcitabine, $p<0.002$, $n=5$). LErafAON treatment (i.v., 25 mg/kg/dose, ×10) caused inhibition of Raf-1 protein expression in these tumor tissues (around 25–60%, $n=4$–7). Interestingly, Taxotere treatment *per se* also led to decreased steady state level of Raf-1 protein in PC-3 and Aspc-1 tumor tissues (i.v., 10 mg/kg/dose, ×1 or 7.5 mg/kg/dose, ×2; around 25–80%, $n=2/6$). Present studies demonstrate enhanced tumor growth inhibition or regression in response to a combination of a chemotherapeutic drug and LErafAON. These data provide a proof-of-principle for the clinical use of LErafAON in combination with chemotherapy for cancer treatment.
***Anti-Cancer Drugs* 15:243–253** © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:243–253

Keywords: chemosensitization, cisplatin, epirubicin, gemcitabine, liposome-entrapped antisense oligonucleotide, mitoxantrone, pancreatic cancer, prostate cancer, Raf-1 inhibition, Taxotere

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Sponsorship: Human prostate and pancreatic cancer cells (PC-3, Aspc-1 and Colo 357) were obtained from the Tissue Culture Shared Resource Facility of the Lombardi Cancer Center (Georgetown University Medical Center). The liposomal formulation reported in this study is patented (US Patent 6,126,965). This study was supported by the NIH grant (PO1 CA74175) and by NeoPharm, Inc.

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Received 11 November 2003 **Accepted** 20 November 2003

Introduction

Raf-1, an approximately 75-kDa cytosolic protein serine/threonine kinase, is an important component of mitogenic and damage-responsive intracellular signal transduction pathways [1,2]. Of particular significance are a number of reports suggesting the roles of Raf-1 in oncogenesis, angiogenesis, metastasis, cell survival, cell-cycle regulation and cytoskeletal reorganization [1–5]. Interestingly, Raf-1 activity can be regulated differently under different physiological or stress-related conditions via a site-specific phosphorylation(s). Mitogen-stimulated phosphorylation of amino acid residue serine 338 (e.g. by p21-activated kinase 1, PAK1 via oncogenic K-Ras) or tyrosine 341 (e.g. by Src) causes Raf-1 activation, whereas phosphorylation of Raf-1 on serine 259 by activated protein kinase A (PKA) or AKT (PKB) results in repression of Raf-1 activity [2]. Consistently, mitogen-induced dephosphorylation of serine 259 by protein phosphatase 2A coincides with activation of membrane-associated Raf-1 [2].

The mechanisms of Raf-1-mediated cell survival are beginning to emerge. The Raf-1–MEK–ERK pathway has been linked with cell survival [6]. Exposure of cells to

0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000119738.70602.15

ionizing radiation stimulates the membrane recruitment, tyrosine phosphorylation and activation of Raf-1 protein kinase [7,8]. Irradiation of cells also activates some of the known downstream effectors of Raf-1 including MEK, ERK and transcription factor, NF-κB [1,7–9]. Raf-1 expression has been associated with inhibition of radiation-induced apoptosis, anti-apoptotic function of phosphatase Cdc25A and restoration of cell-cycle arrest through the p53 pathway [10,11]. Reports using genetic knockouts of the mouse c-*raf*-1 gene demonstrate a MEK-independent role for Raf-1 in cell survival [12,13]. Such mice die of apoptosis and vascular defects. Raf-1 has also been shown to inhibit apoptosis in a MEK–ERK-independent fashion in v-*abl*-transformed cells [14] or by binding to and inhibiting apoptosis signal-regulating kinase 1 (ASK-1) [15,16]. Mutant Raf-1 defective in binding to MEK is capable of inducing NF-κB-mediated gene expression [17]. Furthermore, Raf-1 induces degradation of I-κB, a inhibitor of NF-κB, via MEKK-1, independent of MEK and ERK [18]. Mitochondrial translocation of Raf-1 also appears to play a role in cell survival apparently by inactivation of pro-apoptotic protein BAD and this response is enhanced in conjunction with expression of the anti-apoptotic protein Bcl-2 [19,20]. The PKC-activated mitochondrial Raf-1 appears to be important for anti-apoptotic activity of AKT, which is known to phosphorylate and inactivate BAD [21].

A number of reports suggest Raf-1 as a potential target in cancer chemotherapy. The cytotoxic effects of most chemotherapeutic drugs are mediated by induction of a specific molecular signal(s) and damage to DNA or to the mitotic spindle. Accordingly, defect in a predominant induction of an apoptotic signal or suppression of a survival signal is generally associated with chemoresistance. Raf-1 is vulnerable to damage induced by chemotherapeutic drugs. For example, vinblastine, a microtubule-depolymerizing drug, causes disruption of the mitotic spindle and induces inhibitory phosphorylations of the survival signals Bcl-2 and Raf-1, G_2/M phase arrest, followed by apoptotic cell death [22]. Sensitivity to paclitaxel (Taxol), known to cause microtubule stabilization and G_2/M phase arrest, may be determined by multiple signals including inhibitory phosphorylation of Raf-1 on serine 259, negative regulation of AKT and/or MAPK, p53 status, phosphorylation and degradation of I-κB, phosphorylation and inactivation of members of the Bcl-2 family, and activation of cyclin-dependent kinase (CDK1/p34Cdc2) [23–26]. Raf-1 is a downstream effector for several receptor tyrosine kinases and some of these receptors, e.g. epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (Flk-1), are established targets in cancer chemotherapy [27,28]. Raf-1 also activates the transcription factor NF-κB, in a PAK1-dependent manner, and inhibition of NF-κB causes increased apoptosis in response to chemotherapeutic drugs [29,30]. Constitutively activated Raf-1 substitutes for the growth factor requirements of normal or immortalized cells, and enhances resistance of tumor cells to the toxic effects of ionizing radiation and anticancer drugs, including doxorubicin and paclitaxel [24,31–38]. In addition, the PKC–Raf–MAPK pathway or activation of Raf-1 has been linked with enhanced expression and activity of P-glycoprotein, the product of multidrug resistance gene (MDR1) [37,39,40].

Antisense inhibition of Raf-1 offers a Raf-1 pathway-specific and clinically applicable approach for sensitization of cancer cells to radiation or chemotherapy. Antisense *raf* cDNA transfection or oligonucleotide treatment has been shown to inhibit tumor growth and increase radiation sensitivity of cancer cells [34,41,42]. Selective down-regulation of Raf-1 expression also results in activation of caspase-3, PARP cleavage and apoptosis [43–45]. Previously, we have developed cationic liposomes to deliver ends-modified *raf* antisense oligonucleotides *in vitro and in vivo* [46]. The liposome-entrapped, ends-modified *raf* antisense oligonucleotides (LErafAON) exhibited safety, and favorable pharmacokinetic and biodistribution profiles in mice and monkeys [47]. LErafAON was shown to deliver intact rafAON to tumor tissue, inhibit Raf-1 protein in normal and tumor tissues, and cause radiosensitization *in vivo* in a human hormone-refractory prostate tumor model (PC-3) [47]. These data have led to initiation of phase I clinical trials of LErafAON as a monotherapy and in combination with radiation therapy [48–52]. Here, we examined the antitumor efficacies of combinations of LErafAON and several standard chemotherapeutic drugs. The cytotoxic effects of these drugs have been previously attributed to a variety of different mechanisms. Cisplatin, a platinum-based compound, causes DNA damage via formation of intra- and interstrand adducts with DNA; epirubicin (EPI), an anthracycline structurally related to doxorubicin, intercalates with DNA, inhibiting topoisomerases and repair of DNA strand breaks; mitoxantrone (MTO), an anthraquinone structurally related to doxorubicin, is an inhibitor of topoisomerase II; docetaxel (Taxotere), a taxane, is an inhibitor of depolymerization of microtubules and causes mitotic block; and gemcitabine, an analog of cytosine arabinoside, causes block of DNA synthesis and inhibition of ribonucleotide reductase [53–60]. LErafAON and one of these chemotherapeutic drugs were administered i.v. into athymic mice bearing human prostate (PC-3) or pancreatic tumor xenografts (Aspc-1 and Colo 357). Our second goal was to examine the effect of LErafAON or drug on Raf-1 protein expression in tumor tissues after completion of the treatment. The preclinical data presented provide a proof-of-principle for the clinical use of LErafAON in combination with chemotherapy.

Materials and methods

Oligodeoxyribonucleotides, drugs and chemicals

Antisense *raf* oligodeoxyribonucleotide sequence (5′-GTGCTCCATTGATGC-3′) directed toward the translation initiation site of human c-*raf*-1 mRNA (rafAON) was custom synthesized by Hybridon Specialty Products (Milford, MA). The terminal base linkage at the 5′ and 3′ ends of these sequences was modified to a phosphorothioate group. The sequence of the GMP grade product (97% pure) was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry by the manufacturer. Egg phosphatidylcholine (PC), cholesterol (CHOL) and dimethyldioctadecylammonium bromide (DDAB) were purchased from Avanti Polar Lipids (Alabaster, AL). Alternatively, GMP preparations of lyophilized rafAON and lipids were provided by NeoPharm (Lake Forest, IL). Cisplatin (lot no. 000812) was purchased from Hande Tech Development (Houston, TX). EPI (CAS 56390-09-01, SDS CODE 292) was provided by NeoPharm (Pharmacia & Upjohn, Kalamazoo, MI). MTO hydrochloride (NSC 301739, MSD 5001-07) was provided by the Drug Synthesis & Chemistry Branch, DTP, DCTD, NCI, NIH (Dr Robert J. Schultz). Docetaxel (Taxotere) (NDC 0075-8001-80; Aventis Pharmaceuticals Products) and gemcitabine–HCl (NDC 0002-7501-01, Gemzar; Eli Lilly) were purchased from the Oncology Pharmacy of the Georgetown University Hospital. The drug was freshly reconstituted in sterile normal saline (NS) (1.0 mg/ml cisplatin, EPI, MTO or docetaxel; 6.06 mg/ml gemcitabine). All other chemicals were reagent grade.

Cell cultures

Human prostate carcinoma cells (PC-3) were grown in Improved Minimum Essential Medium (IMEM) (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM glutamine. Human pancreatic carcinoma cells (Aspc-1) were grown in Dulbecco's modified Eagle medium (DMEM) (Biofluids) containing 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM glutamine. Human pancreatic carcinoma cells (Colo 357) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM glutamine. Cell cultures were maintained at 37°C under 95% relative humidity in an atmosphere containing 5% carbon dioxide.

Preparation of LErafAON

The cationic liposomes containing rafAON (LErafAON) were prepared using DDAB, PC and CHOL in a molar ratio of 1:3.2:1.6 as described before [47]. Alternatively, lyophilized lipids supplied by NeoPharm were reconstituted at room temperature with rafAON solution in NS using rafAON to lipid ratio of 1:15 (w/w). The mixture was vortexed vigorously for 2 min, followed by hydration at room temperature for 2 h and sonication for 10 min. LErafAON was stored at 4°C and used within 7 days. Blank liposomes (BL) were prepared exactly as above in the absence of rafAON. These formulations were found to be comparable in encapsulation and efficacy experiments ([47] and present studies).

Animals

Six- to 8-week-old male and female athymic *nu/nu* mice were purchased from the National Cancer Institute (Frederick, MD). The mice were maintained in accordance with the AAALAC accredited procedures in the Research Resource Facility of the Division of Comparative Medicine, Georgetown University Medical Center, and fed purina chow and *water ad libitum*.

Anti-tumor efficacy studies

Therapeutic efficacy of cisplatin, EPI or MTO in combination with LErafAON was determined in the PC-3 human prostate tumor model. Anti-tumor effect of the combination of Taxotere with LErafAON was examined in the human prostate (PC-3) and pancreatic tumor models (Aspc-1 and Colo 357). The efficacy of gemcitabine in combination with LErafAON was examined in the Aspc-1 and Colo 357 pancreatic tumor models. Logarithmically growing PC-3 cells were injected s.c. (5.0×10^6 cells/0.2 ml PBS) in the right flank region of male athymic *nu/nu* mice. Tumors were allowed to grow to a mean tumor volume of greater than 50 mm^3 before treatment initiation (day 0). For Aspc-1 tumor xenografts, logarithmically growing Aspc-1 cells were injected s.c. (2.5×10^6 cells/0.2 ml PBS) in the right flank region of female athymic *nu/nu* mice. Tumors were allowed to grow to a mean tumor volume of greater than 70 mm^3 before treatment initiation (day 0). For Colo 357 tumor xenografts, logarithmically growing Colo 357 cells were injected s.c. (1.0×10^6 cells/0.1 ml PBS) in the right flank region of female athymic mice. Tumors were allowed to grow to a mean tumor volume of greater than 50 mm^3 before treatment initiation (day 0). Tumor-bearing mice were randomly divided into various treatment groups ($n = 5$–8). A desired dose of LErafAON and/or chemotherapeutic drug was administered i.v. via the tail vein at various times. The LErafAON dose and treatment designs were based on our previous experience with the PC-3 tumor model [47] and pilot experiments performed using the pancreatic tumor models. The doses and treatment schedules of chemotherapeutic drugs were based on empirical tests initially conducted in the prostate or pancreatic tumor-bearing athymic *nu/nu* mice. The LErafAON treatment was routinely scheduled at least 4–6 h prior to the drug treatment. Control groups received NS or BL at the same dosing schedule as the experimental groups receiving chemotherapeutic drug or LErafAON, respectively.

The individual tumor sizes in various treatment groups were determined twice a week from caliper measurements of the three major axes (*a*, *b*, *c*) and the tumor volumes were calculated using *abc*/2, an approximation for the volume of an ellipse ($\pi abc/6$). Tumor volume (% initial) was calculated as the percentage of tumor volume prior to the first dosing (day 0; 100%) and the mean tumor volume ± SE (% initial) for each treatment group was plotted. The fold change in initial tumor volume on day *X* was calculated using the formula: (tumor volume on day *X* – initial tumor volume on day 0)/initial tumor volume on day 0 and the mean fold change in initial tumor volume ± SE for each treatment group was plotted.

Raf-1 expression assay

Raf-1 protein expression was analyzed in tumor tissues by immunoblotting with polyclonal anti-Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using standard procedures as detailed earlier [47]. Tumor tissues representing various treatment groups were excised within 24 h of the last LErafAON treatment, followed by immunoblotting of the whole-cell extracts with anti-Raf-1 antibody. The blots were reprobed with anti-G3PDH antibody (Trevigen, Gaithersburg, MD). Relative Raf-1 protein levels were quantified using ImageQuant software (Molecular Dynamics, Eugene, OR).

Statistical analysis

One-way ANOVA was performed to determine the statistical significance of a treatment-related change in mean tumor volume in athymic *nu/nu* mice. $p < 0.05$ was considered to be statistically significant.

Results

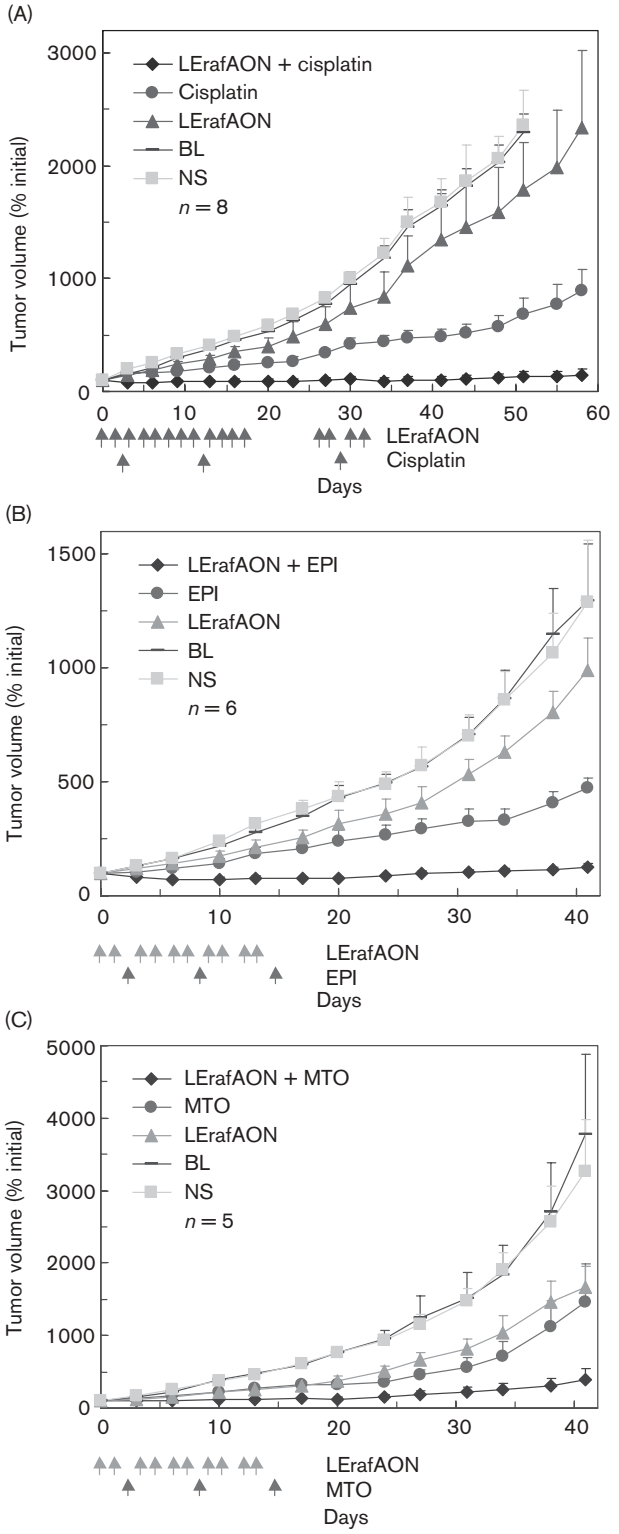
Enhanced anti-tumor effect of combination of LErafAON with cisplatin, EPI or MTO in human prostate carcinoma (PC-3)

Cisplatin treatment alone caused significant tumor growth inhibition as compared with LErafAON alone treatment, and a combination of LErafAON and cisplatin treatments led to a significant tumor growth arrest as compared with single agents. The tumor volumes (% initial) in various treatment groups on day 51, 20 days after the last dosing, were: LErafAON + cisplatin, 129.5 ± 41.6, cisplatin, 680.8 ± 149.8, LErafAON, 1784.0 ± 424.0, BL, 2291.1 ± 167.9, NS, 2365.7 ± 302.4; cisplatin versus LErafAON, $p < 0.005$; LErafAON + cisplatin versus cisplatin or LErafAON, $p < 0.0002$; $n = 8$ (Fig. 1A). All animals in the single treatment groups showed significant increase in initial tumor volumes. In contrast, three of the eight animals in the combination group showed tumor regression and one animal exhibited tumor cure by day 51. The mean increases in initial tumor volumes were significantly lower in the combination group as compared with single agents (day 51: LErafAON + cisplatin, 29%; cisplatin, 581%; LErafAON, 1684%).

The anti-tumor effect of a combination of LErafAON and EPI or MTO was also remarkable as compared with single agents. Epirubicin treatment alone caused significant tumor growth inhibition as compared with LErafAON alone treatment. A combination of LErafAON and EPI treatments resulted in tumor growth arrest, whereas EPI or LErafAON treatment alone caused tumor growth inhibition for up to 26 days after the last dose (day 41: tumor volume, % initial, LErafAON + EPI, 127.5 ± 11.8, EPI, 471.5 ± 48.1, LErafAON, 988.7 ± 141.7, BL, 1296.5 ± 247.5, NS, 1287.2 ± 274.5; EPI versus LErafAON, $p < 0.005$; LErafAON + EPI versus EPI or LErafAON, $p < 0.0001$; $n = 6$) (Fig. 1B). The mean increases in initial tumor volumes on day 41 were: LErafAON + EPI, 27%; EPI, 371%; and LErafAON, 889%. Interestingly, MTO treatment alone caused tumor growth inhibition comparable to LErafAON treatment. However, a combination of LErafAON with MTO caused a significant inhibition of tumor growth as compared with either agent alone (day 38: tumor volume, % initial, LErafAON + MTO, 305.1 ± 103.2, MTO, 1117.4 ± 358.3, LErafAON, 1455.7 ± 300.5, BL, 2706.4 ± 671.1, NS, 2561.9 ± 505.8; MTO versus LErafAON, $p > 0.05$; LErafAON + MTO versus MTO or LErafAON, $p < 0.05$; $n = 5$) (Fig. 1C). The mean increases in initial tumor volumes on day 38

Enhanced anti-tumor effects of a combination of LErafAON and cisplatin, EPI or MTO in human prostate carcinoma. (A) Combination of cisplatin and LErafAON treatments inhibits growth of PC-3 prostate carcinoma in athymic *nu/nu* mice. Tumors were allowed to grow to a mean tumor volume of around 87 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 16; days 0, 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 27, 28, 30 and 31) and/or cisplatin (11.0 mg/kg/dose, × 3; days 2, 11 and 29). Control groups received i.v. either BL or NS on the same schedule as LErafAON or cisplatin, respectively. The study was terminated on day 58. (B) Combination of EPI and LErafAON treatments inhibits growth of PC-3 prostate carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 80 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) and/or EPI (9.0 mg/kg/dose, × 3; days 2, 8 and 15). Control groups received i.v. either BL or NS on the same schedule as LErafAON or EPI, respectively. The study was terminated on day 41. (C) Combination of MTO and LErafAON treatments inhibits growth of PC-3 prostate carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 57 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) and/or MTO (2.5 mg/kg/dose, × 3; days 2, 8 and 15). Control groups received i.v. either BL or NS on the same schedule as LErafAON or MTO, respectively. The study was terminated on day 41.

Fig. 1



were: LErafAON + MTO, 200%; MTO, 1020%; LErafAON, 1360%.

The animals in all treatment groups appeared healthy and no adverse effects were noted for the duration of the study. These results indicate significant anti-tumor effect of a combination of LErafAON and cisplatin, EPI or MTO treatments on PC-3 tumor growth as compared with single agents.

Enhanced anti-tumor effects of combinations of LErafAON and Taxotere in human prostate and pancreatic carcinomas

In the prostate tumor model (PC-3), Taxotere treatment alone caused a significant inhibition of tumor growth as compared with LErafAON alone treatment, and a combination of Taxotere and LErafAON treatments led to tumor regression (day 52: tumor volume, % initial, LErafAON + Taxotere, 70.62 ± 53.45, Taxotere, 417.26 ± 159.55, LErafAON, 1521.17 ± 356.94, BL, 2249.24 ± 369.9, NS, 2256.72 ± 623.36; Taxotere versus LErafAON, $p < 0.005$; LErafAON + Taxotere versus Taxotere or LErafAON, $p < 0.02$; $n = 7$) (Fig. 2A). The Taxotere treatment alone caused tumor regression in one out of seven animals by day 52. In contrast, in the combination treatment group, four out of seven animals showed tumor cure and one animal showed regression of tumor growth by day 52. The mean changes in initial tumor volumes on day 52 were: LErafAON + Taxotere, −29%; Taxotere, 317%; and LErafAON, 1421%. The animals were monitored for up to day 90 and no tumor recurrence was noted in any of the four cured animals in the combination treatment group. These data indicate a high therapeutic efficacy of a combination of LErafAON and Taxotere against PC-3 carcinoma.

The anti-tumor efficacy of a combination of LErafAON and Taxotere was also observed in the pancreatic tumor models (Aspc-1 and Colo 357). The single agents tested (LErafAON or Taxotere) showed comparable and mild effects on Aspc-1 tumor growth as compared with control groups (NS or BL). However, a combination of LErafAON and Taxotere caused a significant inhibition of tumor growth as compared with single agents (day 31: tumor volume, % initial, LErafAON + Taxotere, 199.02 ± 58.76, Taxotere, 575.16 ± 143.29, LErafAON, 594.82 ± 82.77, BL, 840.92 ± 146.49, NS, 842.80 ± 91.81; Taxotere versus LErafAON, $p > 0.05$; LErafAON + Taxotere versus Taxotere or LErafAON, $p < 0.03$; $n = 5$) (Fig. 2B). Furthermore, one out of five tumors in the combination treatment group was cured. The mean increases in initial tumor volumes on day 31 were: LErafAON + Taxotere, 99%; Taxotere, 475%; and LErafAON, 495%.

In the Colo 357 tumor model, Taxotere treatment alone caused a significant tumor growth inhibition as compared

with the LErafAON alone group. A combination of LErafAON and Taxotere caused significant tumor growth arrest as compared with single agents (day 31: tumor volume, % initial, LErafAON + Taxotere, 190.15 ± 52.72, Taxotere, 652.21 ± 259.09, LErafAON, 2096.88 ± 241.17; Taxotere versus LErafAON, $p < 0.0005$; LErafAON + Taxotere versus Taxotere or LErafAON, $p < 0.04$; $n = 7$) (Fig. 2C). The control groups were sacrificed on day 27 in compliance with the tumor burden guidelines (day 27: tumor volume, % initial, BL, 2775.77 ± 956.0, NS, 2846.98 ± 595.96; $n = 7$) (Fig. 2C). The mean increases in initial tumor volumes on day 27 were: LErafAON + Taxotere, 9%; Taxotere, 283%; and LErafAON, 1552%.

The animals appeared healthy and no treatment-related morbidity or mortality was observed for the duration of the study. These results indicate a potent anti-tumor effect of a combination of LErafAON and Taxotere in prostate carcinoma, and an enhanced therapeutic efficacy of this combination in the pancreatic tumor models studied.

Enhanced anti-tumor effects of combinations of LErafAON and gemcitabine in human pancreatic carcinomas

The LErafAON treatment alone appears to be more efficacious in the Aspc-1 tumor model as compared with the gemcitabine treatment alone. A combination of LErafAON and gemcitabine treatments resulted in a significant inhibition of Aspc-1 tumor growth as compared with single agents (day 32: tumor volume, % initial, LErafAON + gemcitabine, 296.3 ± 50.9, gemcitabine, 855.5 ± 48.6, LErafAON, 716.6 ± 67.9, BL, 991.3 ± 97.3, NS, 1037.1 ± 171.1; LErafAON versus gemcitabine, $p < 0.05$; LErafAON + gemcitabine versus gemcitabine or LErafAON, $p < 0.0001$, $n = 7$) (Fig. 3A). The mean increases in initial tumor volumes on day 32 were: LErafAON + gemcitabine, 196%; gemcitabine, 756%; and LErafAON, 617%.

In the Colo 357 tumor model, gemcitabine or LErafAON treatment caused inhibition of tumor growth and these effects were relatively mild as compared with control group (NS or BL). Interestingly, a combination of LErafAON and gemcitabine resulted in a significant inhibition of Colo357 tumor growth as compared with gemcitabine alone but not the LErafAON treatment alone group (day 36: tumor volume, % initial, LErafAON + gemcitabine, 511.1 ± 61.2, gemcitabine, 3112.1 ± 648.1, LErafAON, 1565.0 ± 792.5, BL, 3408.3 ± 625.9, NS, 3476.7 ± 602.7; gemcitabine versus LErafAON, $p > 0.05$; LErafAON + gemcitabine versus gemcitabine, $p < 0.002$; LErafAON + gemcitabine versus LErafAON, $p > 0.1$, $n = 5$) (Fig. 3B). The mean increases in initial tumor volumes on day 36 were: LErafAON + gemcitabine, 411%; gemcitabine, 3012%; and LErafAON, 1465%.

The animals appeared clinically healthy and treatment-related adverse effects were not seen for the duration of the study. These results indicate enhanced anti-tumor activity of a combination of gemcitabine and LErafAON as compared with gemcitabine alone in these two pancreatic carcinoma models.

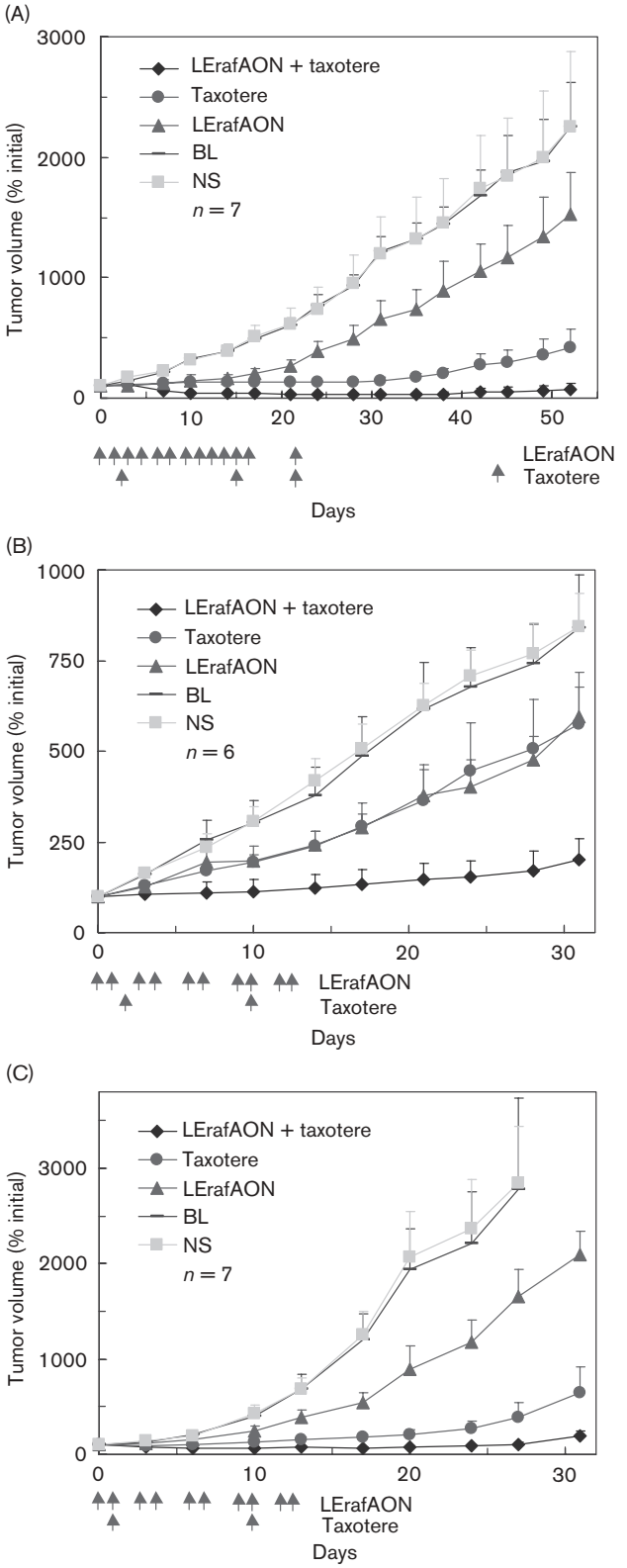
Inhibition of Raf-1 expression in tumor tissues of athymic mice treated with LErafAON or Taxotere

We analyzed the expression of Raf-1 protein in tumor tissues of mice treated with LErafAON or a chemotherapeutic drug. The various drug dosages given were based on the anti-tumor efficacy protocols. Days 0 and 13 corresponded to the first and last days of treatment with LErafAON, and the study was terminated on day 14. LErafAON treatment (25 mg/kg, i.v., × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) led to inhibition of the steady state level of the Raf-1 in tumor tissues as compared to control groups treated with BL or NS (PC-3, ~50%, $n = 5$; Aspc-1, ~25%, $n = 7$; Colo 357, ~60%, $n = 4$) (Fig. 4). These data are consistent with our earlier reports of liposome-entrapped antisense *raf* oligonucleotide-mediated inhibition of Raf-1 protein expression in prostate and head and neck squamous carcinomas [43,47].

No changes in Raf-1 protein expression were identifiable in the tumor tissues collected on day 14 from mice treated i.v. with cisplatin (days 2 and 11; PC-3, $n = 6$), EPI (days 2 and 8; PC-3, $n = 2$), MTO (days 2 and 8;

Chemosensitizing effects of combinations of LErafAON and Taxotere in human prostate and pancreatic carcinomas. (A) Combination of Taxotere and LErafAON treatments causes regression of PC-3 prostate carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 63 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 13; days 0, 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16 and 22) and/or Taxotere (10.0 mg/kg, × 1, day 2; 5.0 mg/kg/dose, × 3, days 15, 22 and 43). Control groups received i.v. either BL or NS on the same schedule as LErafAON or Taxotere, respectively. The study was terminated on day 90. (B) Combination of Taxotere and LErafAON treatments causes growth inhibition of Aspc-1 pancreatic carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 83 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) and/or Taxotere (7.5 mg/kg/dose, × 2; days 2 and 10). Control groups received i.v. either BL or NS on the same schedule as LErafAON or Taxotere, respectively. The study was terminated on day 31. (C) Combination of Taxotere and LErafAON treatments inhibits growth of Colo 357 pancreatic carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 60 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) and/or Taxotere (10.0 mg/kg/dose, × 2; days 1 and 10). Control groups received i.v. either BL or NS on the same schedule as LErafAON or Taxotere, respectively. The study was terminated on day 31.

Fig. 2

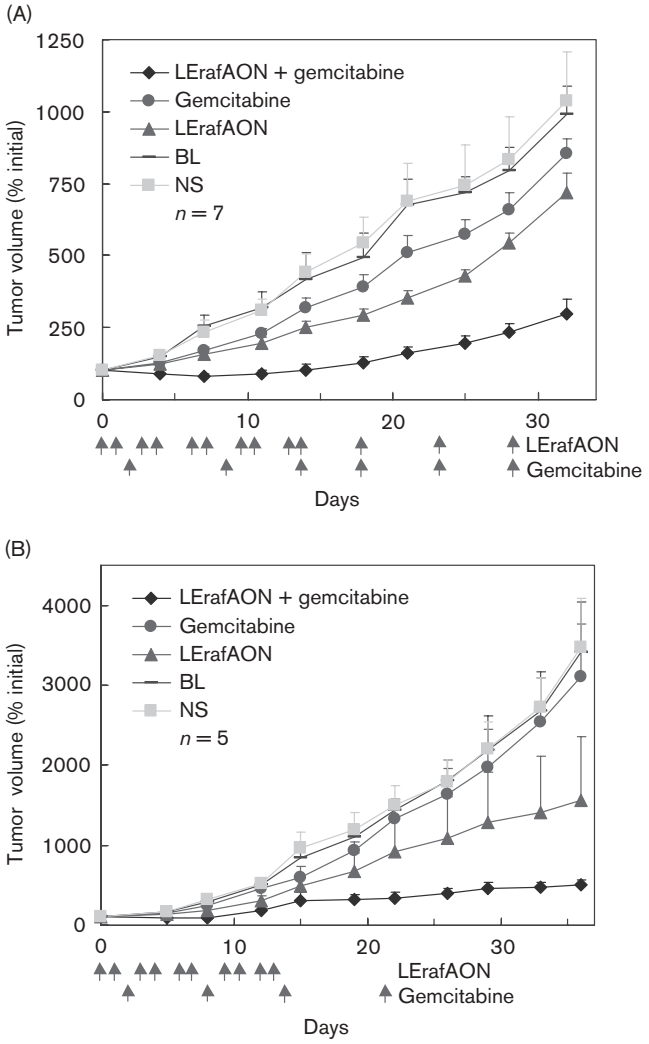


PC-3, $n = 2$) or gemcitabine (days 2, 8 and 13; Aspc-1, $n = 3$) (data not shown). Surprisingly, Taxotere treatment alone caused a prolonged decrease in the steady-state level of Raf-1 protein in the PC-3 and Aspc-1 tumor tissues, but not in the Colo 357 tissues examined (Fig. 5, top panel). These observations are particularly striking because a single dose of Taxotere (10.0 mg/kg, i.v., day 2) led to a noticeable inhibition of the Raf-1 protein level for at least up to 12 days post-treatment in PC-3 tumor tissues ($\sim$25%, $n = 6$). Likewise, a conspicuous inhibition of the Raf-1 protein level was observed for at least up to 4 days after the second dose of Taxotere treatment (7.5 mg/kg, i.v., days 2 and 10) in Aspc-1 tumor tissues ($\sim$80%, $n = 2$) (Fig. 5, bottom panel).

Discussion

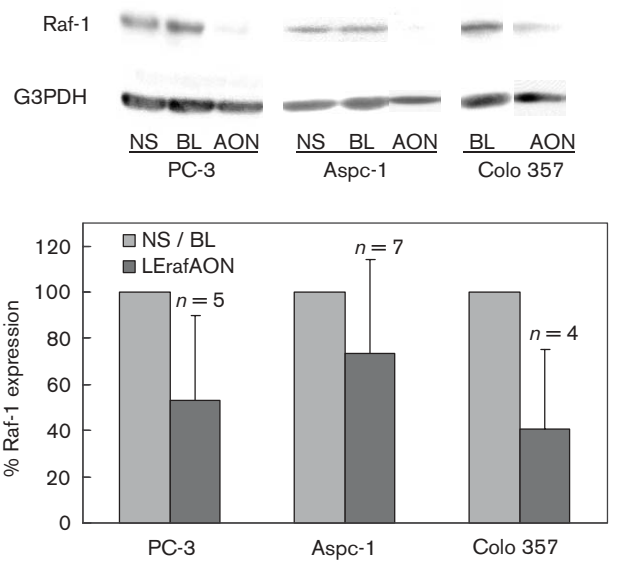
Patients with hormone-refractory prostate cancers (HRPC) may receive single-agent or combination chemotherapy which includes MTO, estramustine, vinblastine, and taxanes such as paclitaxel and docetaxel. Despite advances in the chemotherapeutic management of HRPC, overall survival of patients is approximately 18–24 months [61]. Resistance to chemotherapy and dose-limiting toxicities remain major issues in efforts to improve the survival rates. We show here that anti-tumor activity of a combination of LErafAON and one of the four chemotherapeutic drugs tested is significantly higher as compared to single agents in a HRPC. In the PC-3 tumor xenograft model, cisplatin, EPI or Taxotere treatment alone was more effective as compared to LErafAON treatment alone (Figs 1A and B, and 2A). However, a combination of LErafAON with each of the three drugs resulted in a significantly higher anti-tumor response as compared to the drug alone. Importantly, a combination of Taxotere and LErafAON treatments resulted in tumor cure in four of the seven tumor-bearing mice. The anti-tumor effects of MTO alone were comparable to LErafAON treatment in this tumor model. A combination of MTO and LErafAON treatments resulted in a significant tumor growth inhibition as compared with single agents (Fig. 1C). The effective dose of LErafAON treatment (25 mg/kg, $\times$ 10, i.v.) was also shown to cause 50% inhibition of the steady-state level of Raf-1 protein in tumor tissues excised within 24 h after the last day of treatment ($n = 5$) (Fig. 4). Previously, phosphorothioated antisense *raf* oligonucleotide corresponding to the 3′-untranslated region of c-*raf*-1 mRNA (ISIS 5132) has been shown to enhance antitumor activity of cisplatin against PC-3 tumor xenografts [62]. However, the two important issues of detection of ISIS5132 oligonucleotide and inhibition of Raf-1 expression in tumor tissue have not been addressed in this study. We did not find a change in Raf-1 expression in tumor tissues of mice treated with cisplatin, EPI or MTO treatment alone. It should be noted that these drugs were given once a week over a total of 2 weeks and the tumor

Fig. 3



Enhanced anti-tumor effect of a combination of LErafAON and gemcitabine in human pancreatic carcinoma. (A) Combination of gemcitabine and LErafAON treatments inhibits growth of Aspc-1 pancreatic carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 80 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 13; days 0, 1, 3, 4, 6, 7, 9, 10, 12, 13, 18, 23 and 28) and/or gemcitabine (75.0 mg/kg/dose, × 6; days 2, 8, 13, 18, 23 and 28). Control groups received i.v. either BL or NS on the same schedule as LErafAON or gemcitabine, respectively. The study was terminated on day 32. (B) Combination of gemcitabine and LErafAON treatments inhibits growth of Colo 357 pancreatic carcinoma in athymic *nu/nu* mice. Tumors were grown to a mean tumor volume of around 60 mm^3 and the animals were randomized into five treatment groups. Mice were treated i.v. with LErafAON (25.0 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13) and/or gemcitabine (75.0 mg/kg/dose, × 4; days 2, 8, 14 and 21). Control groups received i.v. either BL or NS on the same schedule as LErafAON or gemcitabine, respectively. The study was terminated on day 36.

Fig. 4

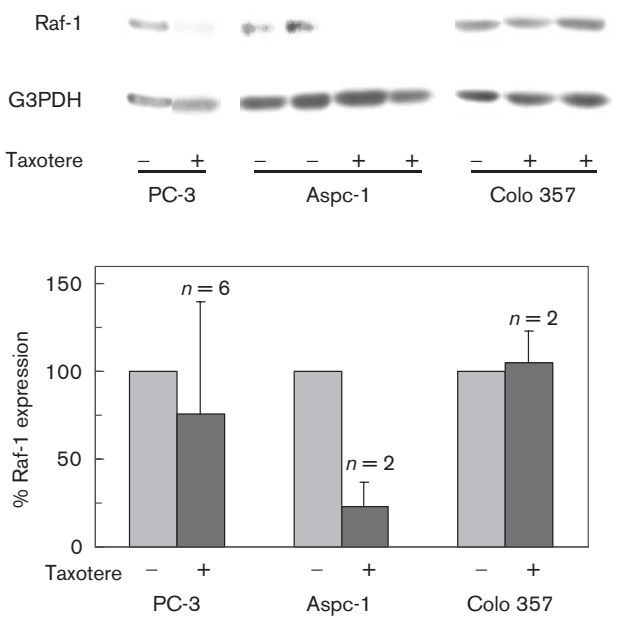


Inhibition of Raf-1 protein expression in tumor tissues of athymic *nu/nu* mice treated with LErafAON. (Top panel) Tumors (PC-3, Aspc-1 and Colo 357) were allowed to grow to around 57–87 mm^3 and the animals were randomized into three treatment groups (LErafAON, BL and NS). Tumor-bearing mice were treated i.v. with LErafAON (25 mg/kg/dose, × 10; days 0, 1, 3, 4, 6, 7, 9, 10, 12 and 13). Control mice received i.v. BL on the same schedule as LErafAON. All mice were humanely sacrificed within 24 h of the last dose and tumor tissues were excised. The Raf-1 expression in tissue homogenates was detected by immunoblotting as explained in Materials and methods. The blots were reprobed with anti-G3PDH antibody as a loading control. Representative data are shown. (Bottom panel) Inhibition of Raf-1 expression in tumor tissues. Quantification data shown are mean ± SD (n = 4–7).

tissues were excised after the second week. In contrast, a single dose of Taxotere (i.v., 10.0 mg/kg) given on day 2 appeared to cause a significantly prolonged inhibition of Raf-1 expression in PC-3 tumor tissues. Approximately, 25% inhibition of Raf-1 expression was noticeable in tumor tissues collected 12 days post-treatment (n = 6) (Fig. 5).

Pancreatic adenocarcinoma, one of the most deadly cancers, is resistant to surgery, radiation and chemotherapy [63]. A majority of the pancreatic tumors overexpress multiple receptor tyrosine kinases, exhibit activated K-*ras* oncogene and NF-κB transcription factor, and K-*ras* and NF-κB are effectors of the Raf-1 signal transduction pathway [64,65]. Gemcitabine is indicated as a first-line therapy in the treatment of pancreatic cancers [66]. Present studies demonstrate significantly increased anti-tumor activity of a combination of LErafAON and Taxotere or gemcitabine as compared with the drug alone in two pancreatic carcinoma models, Aspc-1 and Colo 357 (Figs 2B and C, and 3A and B). The effective dose of LErafAON treatment (25 mg/kg, × 10, i.v.) was also shown to cause around 25% (n = 7) and 60% inhibition (n = 4) of Raf-1 protein expression in Aspc-1 and Colo 357 tumor tissues, respectively (Fig. 4). There was no

Fig. 5



Inhibition of Raf-1 protein expression in PC-3 and Aspc-1 tumor tissues of athymic *nu/nu* mice treated with Taxotere. (Top panel) Tumors (PC-3, Aspc-1 and Colo 357) were allowed to grow to around 57–87 mm^3 and the animals were randomized into two treatment groups (day 0, Taxotere and NS). Tumor-bearing mice were treated i.v. with Taxotere as follows: PC-3, 10.0 mg/kg, day 2; Aspc-1, 7.5 mg/kg, days 2 and 10; Colo 357, 10.0 mg/kg, days 1 and 10. Control mice received i.v. NS on the same schedule as the drug. Mice were humanely sacrificed on day 14 and tumor tissues were excised. The Raf-1 expression in tissue homogenates was detected by immunoblotting as explained in Materials and methods. The blots were reprobed with anti-G3PDH antibody as a loading control. Representative data are shown. (Bottom panel) Inhibition of Raf-1 expression in PC-3 and Aspc-1 tumor tissues. Quantification data shown are mean ± SD ($n = 2/6$).

change in Raf-1 expression in pancreatic tumor tissues of mice treated with gemcitabine alone. Taxotere (i.v., 7.5 mg/kg) treatment given on day 2 and day 10 caused significant inhibition of Raf-1 expression in Aspc-1 tumors ($\sim 80\%$, $n = 2$), but not in Colo 357 tissues (Fig. 5).

The mechanism(s) of chemosensitization *in vivo* as a result of a sustained inhibition of Raf-1 protein expression has not been investigated. However, a number of speculations are plausible. Raf-1 is a survival molecule with established roles in radiation resistance and chemoresistance [1,24,31,34,36–38,67]. Constitutive activation or antisense inhibition of Raf-1 is also associated changes in expression of a large number of known and unknown genes [68–70] (and data not shown). Chemotherapeutic drugs are likely to induce a complex cascade of molecular and biochemical changes associated with cellular protection or cell death response. Indeed, cisplatin, docetaxel and gemcitabine have been shown to induce changes in expression of a myriad of genes [71,72]. Some of these drug-initiated apoptotic pathways may cross-talk or overlap with apoptosis signals stimulated by inhibition of Raf-1. For example, ASK1, a general mediator of cell death, is induced by drugs such as cisplatin and paclitaxel, and inhibited by Raf-1 [15,16]. ASK1 activity is also antagonized by 14-3-3 protein, a known cofactor of Raf-1 [73]. Thus, it is conceivable that inhibition of Raf-1 unmasks the activities of apoptotic signals such as ASK1. In addition, inhibitors of mitogenic kinases may sensitize cancer cells to drug-induced apoptosis [74]. Consistently, the *raf* antisense oligonucleotide-specific inhibition of Raf-1 has been associated with enhanced apoptosis *in vitro* and *in vivo* [43,44]. The biological outcome of signals in response to a combination of a drug and LErafAON is very likely to be determined in cell type- and drug-specific manners. As seen above with docetaxel, a highly potent anti-tumor response can be achieved when Raf-1 itself is a target of the drug. The mechanism of Raf-1 depletion in response to docetaxel is unclear. Native Raf-1 exists as a multimeric complex with hsp90, p50 and other proteins [75]. Previously, the benzoquinone ansamycin geldanamycin has been shown to destabilize Raf-1 by disrupting the native complex [76]. Ongoing studies will address the effects of Taxotere on Raf-1 mRNA and protein stabilities, and post-translational modification. To our knowledge, this is the first report of docetaxel-mediated prolonged inhibition of Raf-1 expression in certain prostate and pancreatic tumor tissues.

The target selectivity and safety issues appear to be the major benefits of a molecular signal-based therapy. Earlier, we have demonstrated a sequence-specific inhibition of Raf-1 protein expression in tumor cells, and in normal and tumor tissues of athymic mice treated with ends-modified *raf* antisense oligonucleotide (rafAON), but not with ends-modified *raf* sense oligonucleotide or ends-modified mismatch antisense *raf* oligonucleotide [42,46,47]. Intact rafAON was detected in normal and tumor tissues of these mice [46,47]. In addition, LErafAON exhibited favorable safety and pharmacokinetic profiles in rodent and primate model systems [47]. The clinical phase I trials of LErafAON as a monotherapy and in combination with radiation therapy have been encouraging [48–52]. Combination chemotherapies are of considerable current interest in the treatment of advanced malignancies [77–79]. Chemotherapeutic agents such as cisplatin, taxanes and gemcitabine have also been shown to enhance the effectiveness of radiation therapy [80]. More recently, a combination of LErafAON, Taxotere, and ionizing radiation was found to be significantly more efficacious as compared with single agents or dual treatments in athymic mice bearing HRPC (data not shown). Present observations provide support of the clinical use of LErafAON in the combined modality strategies for treatment of chemo-refractory cancers.

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