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Halofuginone Enhances the Radiation Sensitivity of Human Tumor Cell Lines

John A. Cook, Rajani Choudhuri, William DeGraff, Janet Gamson, and James B. Mitchell
Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892-1002

Abstract

Transforming growth factor beta (TGF- β) is implicated in radiation-induced fibrosis of normal tissues in patients receiving radiotherapy. Inhibiting the TGF- β signaling pathway by various means has been shown to reduce radiation-induced fibrosis in pre-clinical studies. The present study evaluated the effects of interfering with the TGF- β signaling pathway on the radiosensitivity of selected human tumor cell lines using the plant-derived alkaloid, halofuginone. Halofuginone treatment inhibited cell growth, halted cell cycle progression, decreased radiation-induced DNA damage repair, and decreased TGF- β receptor II protein levels, leading to increased cellular radiosensitization. These data further support the goal of manipulating the TGF- β pathway to achieve a positive increase in the therapeutic gain in clinical radiotherapy.

Keywords

radiation sensitizer; TGF-beta; halofuginone; fibrosis

Introduction

Fibrosis of normal tissues within the treatment field of cancer patients receiving radiotherapy can impair the quality of life for patients. There is considerable evidence implicating the TGF- β signaling pathway as both an initiator and propagator of radiation-induced fibrosis [1-3]. Indeed pre-clinical studies have demonstrated that agents that interfere with radiation-induced TGF- β signaling can lessen radiation-induced fibrosis [4-8]. As agents are developed and evaluated that target members of the TGF- β signaling pathway for potential protection against radiation-induced fibrosis, it is important to also evaluate whether the agents might protect against the radiation effects to the tumor.

We have shown that the plant-derived alkaloid, halofuginone protected against radiation-induced soft tissue fibrosis in a mouse model by virtue of inhibiting various members of the TGF- β signaling pathway [6]. In this study it was also shown that halofuginone did not protect against radiation-induced tumor regrowth delay (murine SCCVII tumor). Encouraged by this

Address correspondence to: James B. Mitchell, Ph.D. Radiation Biology Branch, National Cancer Institute, Bldg. 10, Room B3-B69, Bethesda, MD 20892-1002, Phone: (301) 402-9013, Fax: (301) 480-2238, jbm@helix.nih.gov.

Conflicts of Interest Statement

None Declared

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preclinical therapeutic gain and the lack of tumor protection by halofuginone, it was noted that halofuginone slightly enhanced the tumor response to radiation, though not significantly. This observation prompted the current study to determine if halofuginone might enhance the radiosensitivity of human tumor cell lines.

Materials and Methods

Reagents

Halofuginone (*dl*-trans-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)acetyl]-4(3*H*)-quinazolinone hydrobromide) was obtained from Collgard Biopharmaceuticals, Herzliya, Israel. Stock solutions were prepared by dissolving crystalline halofuginone in a lactic acid buffer, 0.44 M, pH 4.3, and stored at 4°C where it is stable for at least 4 months. Mouse monoclonal anti-phospho histone H2AX (Ser139), clone JBW301 and rabbit antiserum to histone H2A (acidic patch) were purchased from Millipore (Temecula, CA); mouse monoclonal thrombospondin-1 (TSP1) Ab-11 antibody was purchased from Thermo Scientific (Fremont, CA); mouse polyclonal TGF- β type II receptor (TGF- β RII) antibody was purchased from ABNOVA (Taiwan). Mouse monoclonal anti-RAD51C and rabbit polyclonal ATM was purchased from NOVUS Biologicals (Littleton, CO). Mouse polyclonal pATM (Ser1981) was purchased from Cell Signaling (Danvers, MA) and mouse monoclonal HSC70, RIPA lysis buffer as well as all the HRP linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-actin antibody was purchased from Millipore (Temecula, CA), goat anti mouse Alexa Fluor 488 secondary antibody was from Invitrogen (Carlsbad, CA).

Cell Survival Studies

The following cell lines were purchased from American Type Culture Collection (Manassas, VA): MCF7 (human breast carcinoma), A549 (human lung adenocarcinoma), PC3 and DU145 (human prostate carcinoma), and HT29 (human colon carcinoma). PC-Sw and PC-Zd human pancreatic adenocarcinoma cells were obtained from Dr. William Sindelar [9]. Human gastric cancer cell lines, SNU638 and SNU638RII were obtained from Dr. Seong-Jin Kim [10]. All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. For cell survival studies, cells were plated (5×10^5 cells/100 mm plastic petri dish) and incubated for 16 h at 37°C. Halofuginone was then added to the exponentially growing cells (final concentration of 250 nM) in complete medium and the cells were incubated at 37°C for 24 hr. Some studies involved the use of plateau phase cultures. For these studies, cells were allowed to grow to confluence and maintained in confluence without medium change for 3 days after which they were treated with halofuginone as indicated above. Flow cytometry studies confirmed that these cultures were enriched in cells in G1 phase. Following incubation, cells with or without halofuginone, cells were treated with varying doses of radiation using an Eldorado 8 cobalt-60 teletherapy unit (Theratronics International Ltd. Kanata, Ontario, Canada) at dose rates of 2.0-2.5 Gy/min. Control radiation survival curves were conducted in parallel. Immediately after irradiation, cells were trypsinized, counted, plated, and incubated for 10-14 days for macroscopic colony formation. Colonies were then fixed with methanol/acetic acid (3:1) and stained with crystal violet. Colonies with >50 cells were scored and cell survival determined after correcting for the plating efficiency and for halofuginone cytotoxicity alone. For radiation studies, a dose modification factor (DMF) was determined by taking the ratio of radiation doses at the 10% survival level (control radiation dose divided by the drug treated radiation dose). DMF values > 1 indicate enhancement of radiosensitivity.

Growth Rate and Flow Cytometry Studies

The effects of halofuginone on cellular growth rate was determined by plating a number of dishes with same number of cells (6×10^4 cells/dish) and counting the total number of cells

per dish as a function of time (without and with halofuginone, 250 nM). Cell cycle analysis was evaluated by flow cytometry. Following treatment cells were washed with PBS, trypsinized, fixed in cold 70% ethanol diluted in HBSS and stored at 4°C. Cell suspensions were thawed and cells were centrifuged at 1000 rpm for 5 min and the supernatant discarded. The pellet was washed once in cold PBS and suspended in 1 ml of 20 µg/ml of propidium iodide solution containing 0.1% Triton X-100 and 500 ng of DNase free RNase. The cells were immediately analyzed for cell cycle changes using a BD FACS Calibur (BD Biosciences, San Jose, CA).

Western Analysis

Cells were grown to approximately 75% confluence, rinsed with PBS, and switched to medium containing 0.2% serum and incubated overnight. Cells were then treated with halofuginone (250 nM, final concentration) for 24 hr. Following treatment, cells were rinsed with PBS, lysed with RIPA lysis buffer (Santa Cruz Biotechnology) in the presence of sodium orthovanadate and protease inhibitors (Sigma-Aldrich). Following 30 min of incubation on ice, the samples were centrifuged at $14,000 \times g$, and the supernatant protein concentration was determined by DC Protein Assay (BioRad). Protein samples were stored at -70°C for subsequent western analysis. Some studies involved treatment with halofuginone (250 nM) for 24 hr, single dose radiation. After radiation the cells were rinsed twice, fresh medium added, and incubated at 37°C. Protein samples were taken as a function of time after radiation as outlined above.

Protein samples of equal amounts were subjected to PAGE on 4-20% Tris-glycine acrylamide gels (Novex-Invitrogen). Following transfer to nitrocellulose by iBlot Gel Transfer Device (Novex-Invitrogen), samples were probed with primary antibodies diluted 1:200-2000, followed by the appropriate secondary antibody diluted to 1:2000 and visualized by chemiluminescence (PerkinElmer). To confirm equal protein loading and transfer, membranes were stripped by ReBlot Plus (Chemicon) and reprobed using anti-actin antibody. Densitometric analysis was accomplished with image analyzer software coupled with the Fluorchem FC800 system (Alpha Innotech, San Leandro, CA). Density values for each protein were normalized to the actin values.

Results

Halofuginone Enhances the Radiosensitivity of Human Tumor Cell Lines

Pilot studies were conducted to determine the optimal concentration of halofuginone that would enhance a single dose of radiation in PS-Sw cells. It was determined that 24 hr pretreatment with halofuginone at a final concentration of 250 nM provided maximal radiation enhancement (data not shown). For subsequent studies the 24 hr pretreatment and halofuginone concentration was used unless otherwise noted. Figure 1 shows full dose radiation survival curves with and without halofuginone pretreatment for two pancreatic (A, B) and two prostate (C, D) cancer cell lines. Table 1 includes DMF values obtained for the various cell lines. Survival for halofuginone treatment alone was PC-Sw ($54\% \pm 14$); PC-Zd ($84\% \pm 11$); DU145 ($99\% \pm 0$); and PC3 ($39\% \pm 13$). Halofuginone enhanced the radiosensitivity of the pancreatic cancer cell lines to a greater extent than the prostate cancer cell lines with DMF values of 1.6 ± 0.2 and 1.4 ± 0.1 for PC-Sw and PC-Zd cell lines, respectively. DMF values for DU145 and PC3 were 1.4 ± 0.2 and 1.2 ± 0.01 , respectively. Also shown in Table 1 are DMF values for three additional human cancer cell lines. While the radiosensitivity of HT29 cells was enhanced (DMF = 1.3), MCF7 and A549 cells were insensitive to halofuginone-mediated radiation enhancement.

The Effects of Halofuginone on Cell Growth and Cell Cycle Distribution

In order to define possible mechanisms of action of halofuginone PC-Sw cells were used to determine the effects of halofuginone on cell growth. Exponentially growing PC-Sw cells were treated with a final concentration of 250 nM halofuginone and cells were counted as a function of time as shown in Figure 2A. Halofuginone treatment completely inhibited cell growth. To determine if the inhibition in cell growth resulted due to a blockage of cells in a particular phase of the cell cycle, flow cytometry studies were conducted for a 24 hr exposure to halofuginone (250 nM) as shown in Table 2. As can be seen, halofuginone treatment appears to inhibit movement of cells in the cell cycle since there was no accumulation or depletion in G1, S, or G2/M compared to controls. Because halofuginone exerted significant inhibition of cell growth and movement in the cell cycle, the effects of treating cells for 24 hr post-radiation exposure was evaluated to determine if inhibition of cell growth after radiation would modify radiosensitivity (Figure 2B, Table 1). Unlike the radiation enhancement observed for pre-treatment with halofuginone (Figure 1A), post-treatment with halofuginone resulted in no change in the radiosensitivity of PS-Sw cells. To further define halofuginone-mediated enhancement of radiosensitivity and its relationship to cell growth, PC-Sw cells in plateau phase were pre-treated for 24 hr with halofuginone (250 nM), irradiated, and assessed for cell survival (Figure 2C, Table 1). Plateau phase PC-Sw cells consisted of 80.3% G1, 12.8% S, and 6/7% G2/M compared to 41% G1, 50% S, and 8% G2/M for exponentially growing log phase cells. Halofuginone treatment did not radiosensitize plateau phase PC-Sw cells. Collectively, these studies indicate that active cell proliferation is required for halofuginone-mediated radiation enhancement and that the drug be present prior to and not after radiation to exert maximal radiosensitization.

Halofuginone Inhibits the Repair of Radiation-Induced Damage

The effects of halofuginone on radiation-induced repair were monitored by following the induction of phosphorylated γ -H2AX [11]. DU145 and PC-Sw cells were pre-treated with halofuginone for 24 hr (250 nM) at which point the cells were irradiated, rinsed, and incubated in fresh medium at 37°C. Proteins for phosphorylated γ -H2AX western analysis were collected as a function of time after radiation. Figure 3 shows that for control cells 1 hr post-radiation there is a substantial increase in phosphorylated γ -H2AX for both cell lines, followed by a steady decrease in phosphorylated γ -H2AX levels at 6 and 24 hr post-radiation indicating repair of radiation DNA damage. Halofuginone pre-treatment slightly increased the level of phosphorylated γ -H2AX levels in both cell lines; however, at 6 and 24 hr post-radiation phosphorylated γ -H2AX levels remained elevated compared to controls indicating inhibition of radiation-induced repair. In fact halofuginone treatment alone resulted in elevated levels of phosphorylated γ -H2AX at 24 hr, suggesting that halofuginone was inducing a DNA damage/repair response. Induction of γ -H2AX following a 24 hr exposure to halofuginone (250 nM) alone was also observed in MCF7 and HT29 cells (data not shown). To further study this observation two cell lines radio-enhanced by halofuginone (DU145, PC-Sw) and two cell lines minimally enhanced by halofuginone (MCF7, HT29) were treated with halofuginone alone for 24 hr (250 nM) and proteins collected to determine the induction of phosphorylated ATM and Rad51C. These proteins were selected because they are key players in two different DNA repair pathways, non-homologous end joining (NHEJ) [12] and homologous recombination (HR) [13]. Figure 4 shows that halofuginone treatment induced phosphorylated ATM in the two cell lines that were radiosensitized by halofuginone (DU145 and PC-Sw); whereas, the levels of phosphorylated ATM were unchanged in MCF7 and HT29 cells, which showed minimal enhancement of radiosensitivity. With exception to a small decrease as a result of halofuginone treatment in DU145 cells, halofuginone had minimal effects on Rad51C levels for the other three cell lines.

Halofuginone Effects on Signaling Pathways

A brief survey was conducted to determine the effects of halofuginone treatment on selected signaling pathways including EGFR and IGF1R β . These pathways were selected because inhibition of the pathways has been associated with enhancement in radiation response [14, 15]. MCF7, HT29, PC-Sw, and DU145 were exposed to halofuginone (250 nM) for 24 hr and proteins collected for western analysis. Halofuginone treatment had no effect on EGFR or IGF1R β for any of the cell lines (data not shown). We next explored the effects of halofuginone on the TGF- β signaling pathway. One of the upstream molecules in the TGF- β signaling pathway is the TGF- β RII receptor [16]. Figure 5A shows the effects of a 24 hr treatment with halofuginone (250 nM) on TGF- β RII levels in the same four cell lines listed above. Halofuginone treatment suppressed TGF- β RII levels in HT29, PC-Sw, and DU145 cells. It is interesting to note that all three of these cell lines were radiosensitized by halofuginone treatment (Table 1). The MCF7 cell line was not radiosensitized by halofuginone and had relatively low levels of TGF- β RII, which were unaffected by halofuginone treatment. To further evaluate the potential role of TGF- β RII suppression by halofuginone with respect to radiosensitivity, a pair of cell lines were evaluated that either lack functional TGF- β RII protein (SNU-638) or have a wild type TGF- β RII gene re-inserted (SNU-638RII) [10]. Figure 5B shows that the SNU-638 is devoid of TGF- β RII protein, while the SNU-638RII cell line has considerable TGF- β RII protein that is markedly suppressed upon halofuginone treatment. Figure 5C and D show radiation survival curves for SNU-638 and SNU-638RII exposed to a 24 hr pretreatment with halofuginone (250 nM) before radiation treatment. Halofuginone treatment had no effect on SNU-638 cells; however, SNU-638RII cells were radiosensitized with a DMF of 1.4 ± 0.1 ($p < 0.01$) (Table 1). These data suggest that suppression of the TGF- β signaling pathway is involved in halofuginone-mediated radiation sensitization.

Discussion

Our initial interest in inhibiting the TGF- β signaling pathway came from studies, which demonstrated that radiation-induced skin damage and early fibrosis was attenuated in Smad3 knockout mice compared to wild type mice [17]. This study strongly suggested that inhibition of the normal TGF- β signaling pathway may be beneficial in preventing or lessening radiation-induced fibrosis, an untoward toxicity associated with radiotherapy. There is considerable evidence that suggests that activation of the TGF- β pathway is a major contributor to radiation-induced fibrosis [1-3].

Halofuginone is a plant-derived alkaloid that exhibits inhibition of type I collagen synthesis, anti-fibrotic activity, anti-angiogenic activity, and anti-tumor activity [18-24]. The breadth of effects exerted by halofuginone is thought to reside in part from halofuginone's impact on the TGF- β signaling pathway [24]. Halofuginone has been shown to inhibit TGF- β dependent Smad3 phosphorylation, inhibit TGF- β RII, and elevate inhibitory Smad7 in a variety of cell types [6,25,26]. Pre-clinical studies have shown that halofuginone protected against radiation-induced soft tissue fibrosis by virtue of inhibiting various members of the TGF- β signaling pathway [6]. Other agents have been used to target the TGF- β signaling pathway and have shown efficacy in pre-clinical models where radiation-induced fibrosis is decreased [4,5,7,8]. In all of these studies it is of utmost importance to determine if the agents afford protection against radiation-induced tumor response, since their intended use would be in a radiation oncology setting. Since halofuginone has documented anti-tumor effects [24], it would be anticipated that it would not interfere with radiation-induced tumor regrowth delay. Indeed, halofuginone did not protect against radiation-induced tumor regrowth delay for the murine tumor SCCVII [6]. The current study extends this observation to include several human tumor cell lines, which are in fact sensitized as a result of halofuginone treatment.

The radiosensitivity of pancreatic, prostate, and colon carcinoma cell lines was enhanced by pretreatment with halofuginone. Although not an exhaustive survey, halofuginone enhanced the radiosensitivity of cell lines that were mutant for p53 (PC-Sw, PC-Zd, DU145, PC-3, and HT29) to a greater extent than p53 wild type (MCF7 and A549) cells (Table 1). This is interesting because it suggests involvement of p53 with the TGF- β signaling pathway [27]. Halofuginone-mediated enhancement of radiosensitivity depended heavily on the timing of halofuginone treatment, where pre-treatment with halofuginone enhanced radiosensitivity and post-treatment did not. Enhancement of radiosensitivity by various agents is often accompanied by alteration in cell cycle distribution to more radiosensitive stages of the cell cycle (G2/M). Halofuginone treatment inhibited cell growth by virtue of halting cell cycle progression in all phases of the cell cycle. This finding is unique and the mechanism of this action is not clear. It is clear however, that halofuginone-mediated radiation enhancement requires an actively dividing population of cells initially because plateau phase cells, which are enriched in non-cycling G1 cells were not radiosensitized by halofuginone.

Yet another factor that can contribute to radiosensitization is inhibition of the repair of radiation-induced damage. Studies using γ -H2AX indicate that halofuginone inhibits radiation-induced DNA damage repair; however, a complicating feature of the studies was that halofuginone treatment alone induced γ -H2AX. Likewise, halofuginone treatment alone induced pATM (Figure 4), consistent with a DNA strand break damage response. However, since halofuginone treatment was capable of halting cell cycle progression, it is possible that halofuginone interfered with normal DNA replication thereby inducing replication stress, which has been shown to mediate γ -H2AX induction [28]. Induction of γ -H2AX has been demonstrated for cells treated with gemcitabine and arrested in S-phase [29,30]. A signature of replication stress is a pan nuclear γ -H2AX staining of the entire nucleus uniformly [31]. In contrast, halofuginone treatment alone showed distinct foci formation in the nucleus of PC-Sw cells (data not shown) and in agreement with pATM activation suggesting a DNA damage response. Since halofuginone has demonstrated anti-tumor activity [22,32-34] it will be important to determine the contribution of halofuginone-mediated γ -H2AX induction either from DNA damage response versus replication stress on cytotoxicity and radiation enhancement.

Lastly, another approach that has been shown to enhance the radiosensitivity of cancer cells is through interfering with various signaling pathways. Examples include EGFR and IGF1R, where inhibition of these pathways by various means results in enhancement of radiosensitivity [14,15]. We observed no alteration of EGFR and IGF1R protein levels following halofuginone treatment in PC-Sw cells (data not shown). However, halofuginone is known to influence TGF- β signaling [6,19]. Indeed halofuginone treatment decreased TGF- β -RII protein levels (Figure 5) and enhanced radiosensitivity (Table 1) in HT29, PC-Sw, and DU145 cells; whereas, TGF- β -RII protein levels were unchanged in MCF7 with no enhancement in radiosensitivity. To further study the effects TGF- β -RII protein levels on radiosensitivity, SNU-638 cells were used. These cells have a DNA replication error phenotype that encodes for a truncated and inactive TGF- β -RII protein. Transfection of these cells (SNU-638RII) with retroviral vectors containing wild type TGF- β -RII leads to restoration of normal TGF- β -RII protein [10]. As shown in Figure 5B SNU-638 cells exhibit no TGF- β -RII protein; however, the restored cell line, SNU-638RII displays significant levels of TGF- β -RII, which was markedly depleted following halofuginone treatment. SNU-638 cells were not radiosensitized by halofuginone treatment (Figure 5C); whereas, the radiosensitivity of SNU-638RII cells was enhanced by halofuginone (Figure 5D). These data suggest that interfering with the TGF- β signaling pathway can impact radiosensitivity, particularly with a signaling member that is upstream in the pathway.

Andarawewa et al. suggest that inhibition of the TGF- β signaling pathway may lead to enhanced radiation sensitivity of tumor cells and protection against radiation-induced fibrosis [3,35]. The findings of the present study for tumor cell radiosensitization, coupled with our previous study showing protection of radiation-induced fibrosis in normal tissue [6] supports this concept.

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Figure 1. Radiation survival curves for cell lines pre-treated with halofuginone (250 nM) for 24 hr (closed circles) or without halofuginone treatment (open circles). **A)** PC-Sw, **B)** PC-Zd, **C)** DU145, **D)** PC3.

Figure 2.

A) PC-Sw cell growth for control (open circles) or cells treated with halofuginone (250 nM) as a function of time. **B)** Radiation survival curves for PC-Sw treated with halofuginone (250 nM) after radiation for 24 hr (closed circles) or without halofuginone treatment (open circles). **C)** Radiation survival curves for plateau phase PC-Sw cells pre-treated with halofuginone (250 nM) for 24 hr (closed circles) or without halofuginone treatment (open circles).

A) DU-145

Time (hr)	1	1	6	24	24	1	6	24
X	-	+	+	+	-	+	+	+
HF	-	-	-	-	+	+	+	+

B) PC-Sw

Time (hr)	1	1	6	24	24	1	6	24
X	-	+	+	+	-	+	+	+
HF	-	-	-	-	+	+	+	+

Figure 3.

Western analysis for phosphorylated γ H2AX as a function of time following pretreatment with halofuginone (250 nM) for 24 hr and single dose radiation. **A)** DU145 cells (radiation dose = 5 Gy) and **B)** PC-Sw (radiation dose = 8 Gy). Normalized density levels of protein bands for each study are shown below the respective westerns using H2A as the loading control.

Figure 4. Western analysis for pATM and Rad51C proteins following treatment of MCF7, HT29, PC-Sw and DU145 cells with halofuginone (250 nM) for 24 hr. Loading controls were ATM and β-actin.

Figure 5.

A) Western analysis for TGF- β RII protein following treatment of MCF7, HT29, PC-Sw and DU145 cells with halofuginone (250 nM) for 24 hr. Loading control was HSC90. **B)** Western analysis for TGF- β RII protein following treatment of SNU-638 and SNU-638RII cells with halofuginone (250 nM) for 24 hr. Loading control was HSC70. Radiation survival curves for SNU-638 (**C**) and SNU-638RII (**D**) cell lines pre-treated with halofuginone (250 nM) for 24 hr (open circles) or without halofuginone treatment (closed circles).

Table 1

Halofuginone Enhancement of Radiosensitivity for Selected Human Tumor Cell Lines

Cell Line	DMF*
PC-Sw	1.6 ± 0.2
PC-Zd	1.4 ± 0.1
DU145	1.4 ± 0.2
PC3	1.2 ± 0.01
HT29	1.3
MCF7	1.0 ± 0.2
A549	1.1
SNU-638	1.1 ± 0.1
SNU-638RII	1.4 ± 0.1
PC-Sw (post-tx)	1.1 ± 0.1
PC-Sw (plateau)	1.0 ± 0.1

* Mean DMF ± SEM

Table 2

Cell Cycle Analysis of Control and Halofuginone Treated PC-Sw Cells

Condition	G1 (%)	S (%)	G2/M (%)
Control 24 hr	44 ± 0.41	43 ± 0.80	13 ± 0.72
HF (250 nM) 24 hr	38 ± 0.58	42 ± 1.20	19 ± 1.05

Values represent the mean ± SEM of 3 independent experiments