NKH477 INHIBITS PROLIFERATION AND INDUCES APOPTOSIS IN A PANEL OF CANCER CELL LINES

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ABSTRACT

Background and Objectives: *The anti-proliferative effects of cAMP in cancer cells may be regulated by the adenylyl-cyclase-V isoform. As Colforsin Daropate (NKH477) is more selective for this isoform, we hypothesized that this water soluble compound may promise utility as an oral anti-tumour agent.*

Materials and Methods: *Using separate cancer cell lines (MCF7, HT29, A431, WiDr, RKO, A375, H630, Du145, SW480 and SW620), we studied the effects of NKH477 on cell proliferation, cell viability and apoptosis.*

Results: *NKH477 induced >70% inhibition of proliferation in all cancer cell lines tested. NKH477 induced a dose-dependent apoptosis causing G1 arrest and priming cells to die.*

Conclusions*: NKH477 treatment on the tested cell lines inhibited proliferation and induced apoptosis. Thus, NKH477 shows early promise as an oral anti-cancer agent.*

Key word: *NKH477, Apoptosis, cAMP, Cancer*

INTRODUCTION

Cyclic AMP (cAMP) and cyclic GMP (cGMP) signaling are important nucleotide regulators of crucial cellular activities such as proliferation, growth, migration, metabolism, and apoptosis. Cancer perturbs each of these processes[1, 2]. cAMP dependent protein kinase and protein kinase A (PKA) act as effectors capable of eliciting cellular responses via transduction of intracellular cAMP[2-4]. Other effectors include camp dependent guanine nucleotide exchange factor [cAMP-GEFs] and exchange protein activated by camp [Epac] (which regulates the small GTPase Rap1[5, 6], or cAMP gated ion channels)[1]. Within the past 10 years another cAMP effector molecule has been identified, during a database search of cAMP effectors containing second messenger binding motifs[5-7]. The aim of this study was to address the generality of NKH477 (Colforsin Daropate) treatment to inhibit proliferation of cancer cell lines of different origin.

MATERIALS AND METHODS

Cells & Reagents

Cell culture media for the cell lines used in this study include: MCF7 as per Fidler model cells; HT29, A431, WiDr, RKO, A375, H630, Du145 were all cultured in DMEM supplemented with 10% FBS and 1% L-gluatamine; SW480 and SW620 were cultured in RPMI supplemented with 10% FBS and 1% L-glutamine. All cells were routinely maintained in an humidified incubator at 37ºC with 5% CO2 and sub-cultured prior to reaching confluence. All sub-culturing of adherent cells was achieved by removing the medium, and then by aspiration with the monolayer rinsed with PBS, then with 10% trypsin/PE solution. Upon detachment, the cells were resuspended in

appropriate media, counted, and then transferred into tissue culture flasks or plates. Cells were generally treated one day after plating. NKH477 (Colforsin Daropate Hydrochloride) and the cell lines were a kind gift from Dr. Mohamed Al-Rabea (Um Al-Qura University, Makkah, KSA).

Treatment of cells

Cells were treated with the numerous chemical compounds at the concentrations and time points indicated in each Figure legend.

MTT proliferation assay

Cell proliferation and viability was assayed indirectly by a modified MTT assay, based on the enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to formazan crystal by mitochondria and cellular dehydrogenase enzymes[8].

Annexin V detection of apoptosis

Apoptosis was quantified using an Annexin V-FITC detection kit (Beckton Dickinson) and staining was carried out as per manufacturer's instructions. Cells were analysed using a Beckton Dickinson FACScan flow cytometer (Oxford, UK).

Determination of apoptotic cells by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) in tested cell lines

Tested cell lines resuspended in Dulbecco's MEM (Gibco) were mixed with equal volumes of 8% paraformaldehyde for 10 minutes. The cells were pelleted at 1800 rpm for 5 minutes then resuspended in Dulbecco's MEM (Gibco) at a concentration of $2x10^5$/ml and 100 µl volumes cytospun on to cleaned microscope slides at 450 rpm for 10 minutes (Shandon Cytospin 2;Shandon, Pittsburgh, PA, USA). Slides were air dried overnight, rehydrated in TBS for 15 minutes at room temperature and dried. The cells were covered by a 5 ml droplet of protein K diluted 1:100 in 10mM Tris (pH 8), incubated 5 minutes at room temperature then dipped three times into TBS and dried. The specimen was covered with 100 µl of supplied equilibration buffer and incubated for 30 minutes at RT. Excess buffer was poured off and freshly prepared TdT labelling mixture (3µl TdT enzyme in 57µl TdT labelling reaction mix (Frag EL:Calbiochem, Nottingham, UK) was layered on to the cells. The slide was incubated at 37C in humidified chamber for 1.5 hour then washed x3 in TBS at room temperature. A coverslip was applied over mounting medium (Frag EL) and sealed with nail varnish to prevent evaporation. At least 500 cells from randomly selected fields were scored by fluorescent light microscopy (494 nm). Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies staining bright green.

RESULTS

NKH477 inhibits the proliferation of cancer cell lines

Proliferation of colon cancer cell lines (H630, HT29, RKO, WiDr, SW480 and SW620), squamous cell carcinoma (SCC; A431), prostate (Du145), breast (MCF7) and skin melanoma (A375) cell lines was assessed after 72 hours treatment with either control (DMSO), 100ìM NKH477 using an MTT assay and was expressed as a percentage of control. 100 µM NKH477 concentrations inhibited all the cell lines to >82% of control.

We assessed the effects of the NKH477 on proliferation of the cancer cell lines described previously. In this case, treatment with either 100 or 50 µM NKH477 alone induced approximately 70% (or greater) inhibition in all of the 10 cell lines tested (Figure-2). Thus, all 10 cell cancer cell lines tested were highly sensitive to NKH477.

NKH477 induces apoptosis and DNA fragmentation

The process of apoptotic cell death is characterised by several morphological and biochemical features that allows identification of dying cells in a population. Morphological features include cell shrinkage, rounding and blebbing of the membrane. Cells were seeded at a density of 5 x 10^5 cells per 60mm dish, allowed to adhere overnight and treated with the standard conditions. In the control cultures after 48 hours, cells were tightly aggregated. However, 'high dose' NKH477 treatment resulted in fewer of these colonies and the emergence of what appeared to be small, round and shrivelled cells (data not shown). 'Low dose' NKH477 had only a partial effect had no effect on the morphology of the cells. This data suggested that 'high dose' NKH477 may have been inducing some form of cell death which would account for the differences observed in the proliferation assays (Figure-1).

To quantify the relative abilities of each standard treatment used to induce apoptosis, cells were seeded and treated for 24, 48 or 72 hours, fixed in ethanol, stained with Annexin-V, and analysed by flow cytometry. Fragmented DNA appears as the sub-2n-DNA region of the histograms (arrow indicates regions of interest (data not shown). Cells were trypsinised and stained by TUNEL assay. Figure-2 shows that 100µM NKH477 induced a high frequency of apoptotic cells. The frequency of apoptotic cells

was reduced significantly in the control alone (p=0.05) (data not shown).

SCC cells were incubated in the presence of 0, 10µM, 50µM, and 100µM NKH477 for two days stained with TUNEL assay. Data is a representative of three experiments.

We analysed intact cells, early apoptotic and late apoptotic cells. Scatter plots were generated (Figure-3) and the lower right quadrants (corresponding to early apoptotic cells) were quantified. Cells treated with either 'high dose' NKH477 were induced to die by apoptosis (26% and 27% of gated cells respectively at 72 hours). This became statistically significant at 48 hours ($P < 0.05$) when compared to 'low dose' NKH477 (Figure-3). Control treated cells did not show a significant induction of apoptosis after 72 hours (Figure-3). Importantly, 'low dose' NKH477 produced only a partial induction of early apoptotic cells (Figure-3).

To confirm the type of cell death induced cells were treated with with control (DMSO), 1 µM NKH477, 10 µM NKH477, 50 µM NKH477 for 24, 48 and 72 hours. SCC Cells were then washed, trypsinised and incubated with Annexin-V FITC conjugate, PI and analysed by flow cytometry for the detection of early apoptotic cells. Representative scatter plots for all time points and treatments are shown, with the lower right quadrant (corresponding to early apoptotic cells) indicated by the arrow.

Figure 1: NKH477 inhibits the proliferation of all cancer cell lines

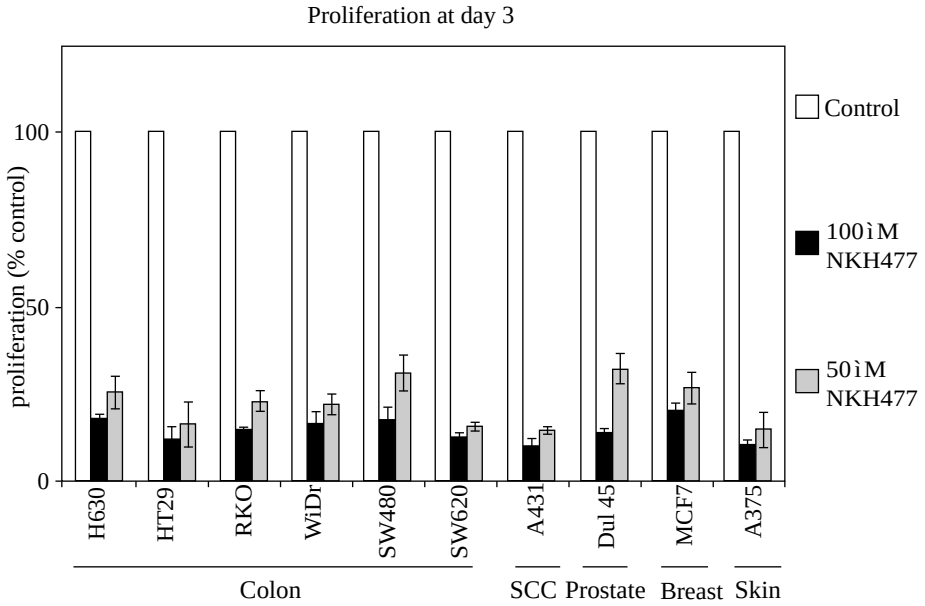


Figure 2: NKH477 induced DNA fragmentation

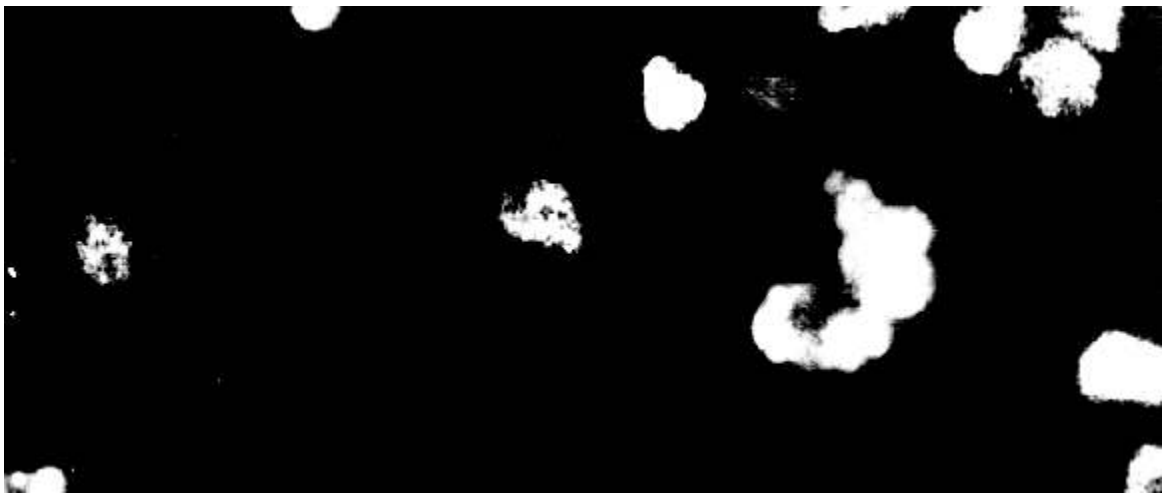
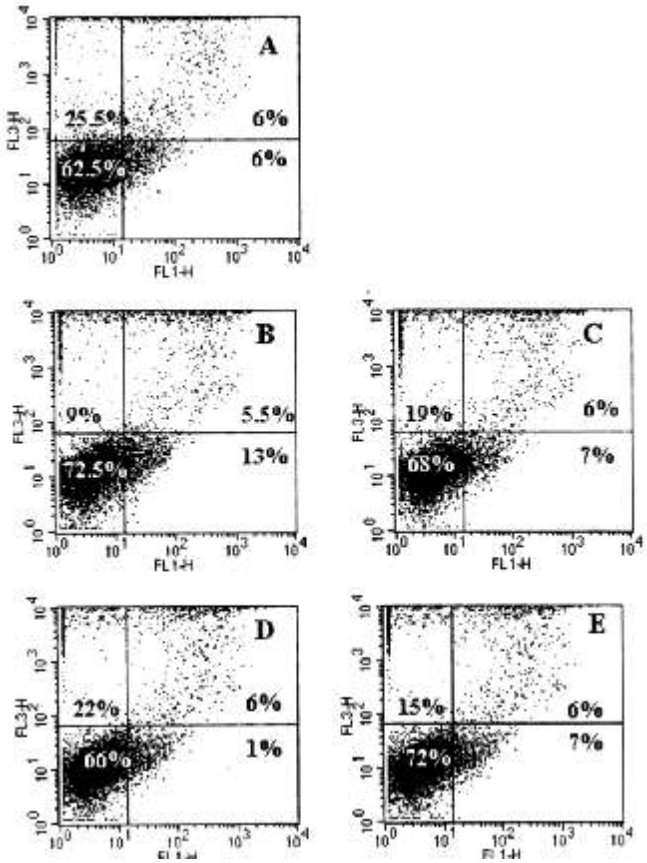


Figure 3: Analysis of NKH477-induced apoptosis in SCC cells after 3 days



A Control

B 100 µl NKH477

C 50 µl NKH477

D 10 µl NKH477

E 1 µl NKH477

Quadrant percentages are marked on each plot and represent as follows:

Lower left quadrant: Cells which do not stain for either propidium iodide or annexin V and are not undergoing apoptosis,

Lower right quadrant: Cells which stain positive for annexin V only and are undergoing early apoptosis,

Upper right quadrant: Cells which stain positive for both markers and are in late apoptosis,

Upper left quadrant: Cells which stain positive for propidium iodide only and represent dead cells. These are data are a representative of 3 experiments.

DISCUSSION

A range of genetic inactivations and mutations in mature cancer cells typically underpin the nature and aggressiveness of individual tumours. There are many examples of this. EGFR is often overexpressed in lung cancer leading to hyperactivation of ERK and PI 3-kinase pathways. Mutations in the PI 3-kinase pathway commonly occur in colon cancer[9] (and involve inactivation of PTEN or activating mutations in p85 or p110, as well as loss of APC). Frequently in skin cancer, hyperactivation of the ERK pathway may caused by a common activating mutation (B-Raf V600E)[10]. In our study, we assessed the sensitivity of a variety of cancer cell lines to NKH 477 mediated inhibition of proliferation and found that there were distinct variations in their sensitivities.

In seeking cancer treatments that can safely be used clinically, it is important to identify (typically small) molecules which exhibit no adverse toxic events and that also meet criteria for solubility and bioavailability. NKH477 (or Colforsin Daropate hydrochloride) was therefore developed to provide a water soluble analogue more suitable for in vivo use[11]. NKH477 has been used in a wide variety of research models which include studying immuno-modulation in mice[12], ischemia[13] and arrhythmias[14] in dogs, and an anti-

depressant model in rats[15]. Clinically, NKH477 has also been used in humans; as a bronchodilator[16, 17], as an immune-suppressant, and as a vasodilator during cardiac surgery[18]. To date however, there have been no reports using this compound in an anti-cancer context either in cell research, or in animal or human based studies.

In our study, we showed that NKH477 suppressed the proliferation of all 10 cell lines tested. We have also shown that NKH477 suppresses the proliferation of KM12C cells, and that a low concentration of NKH477 was also able to synergise with rolipram (data not shown). The mechanisms involved in NKH477-mediated inhibition of proliferation in the panel of cell lines we used remain unclear.

It has been reported that NKH477 is more selective for the adenylyl cyclase-V isoform[19] and that could well be the isoform important in the regulation of the anti-proliferative effects of cAMP in cancer cells. These data suggests the exciting possibility that this orally available, water soluble, compound may be useful as an anti-cancer therapy.

In conclusion, it must be emphasized that tested cell lines are not the same as human cancer cells. This raises the exciting possibility of using NKH477, a compound which has extensively been tested in with no adverse effects, as a potent anti-cancer agent. Additionally, Further work to elucidate the mechanisms by which NKH477 so potently inhibits variety of animal and human cancer tissues is required if any potential anti-cancer therapies based around this drug are to be realized.

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