MOL(13474)

Vitamin K3 (menadione) induced oncosis associated with keratin 8 phosphorylation and histone H3 arylation

Gary K. Scott, Christian Atsriku, Patrick Kaminker, Jason Held, Brad Gibson, Michael A. Baldwin and Christopher C. Benz

Buck Institute for Age Research (GKS, CA, PK, JH, BG, MAB, CCB)

Department of Pharmaceutical Chemistry, University of California, San Francisco (CA, BG, MAB)
Menadione induced modifications to keratin 8 and histone H3

Corresponding author: Christopher C. Benz, MD

Program of Cancer and Developmental Therapeutics, Buck Institute for Age Research
8001 Redwood Blvd., Novato, CA 94945
tel #: 415-209-2092; fax #: 415-209-2232
email: cbenz@buckinstitute.org

Number of text pages: 19
Number of tables: 0
Number of figures: 8
Number of references: 30
Number of words in Abstract: 241
Number of words in Introduction: 542
Number of words in Discussion: 1,259

Non-standard abbreviations: K3, menadione or vitamin K3; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; k-8, keratin 8; MAPK/Erk, mitogen activated protein kinase/extracellular regulated kinase; ROS, reactive oxygen species; NAC, N-acetyl cysteine; IAA, iodoacetic acid; LC-ESI_MS liquid chromatography electrospray ionization mass spectrometry
Abstract

The vitamin K analog menadione (K3), capable of both redox cycling and arylating nucleophilic substrates by Michael addition, has been extensively studied as a model stress inducing quinone in both cell culture and animal model systems. Exposure of keratin 8 (k-8) expressing human breast cancer cells (MCF7, T47D, SKBr3) to K3 (50-100µM) induced rapid, sustained and site-specific k-8 serine phosphorylation (pSer73) dependent on signaling by a single mitogen activated protein kinase (MAPK) pathway, MEK1/2. Normal nuclear morphology and k-8 immunofluorescence coupled with the lack of DNA laddering or other features of apoptosis indicated that K3 induced cytotoxicity, evident within 4 h of treatment and delayed but not prevented by MEK1/2 inhibition, was due to a form of stress activated cell death known as oncosis. Independent of MAPK signaling was the progressive appearance of K3 induced cellular fluorescence, principally nuclear in origin and suggested by in vitro fluorimetry to be due to K3 thiol arylation. Imaging by UV transillumination of protein gels containing nuclear extracts from K3 treated cells revealed a prominent 17 kDa band shown to be histone H3 by immunoblotting and mass spectrometry (MS). K3 arylation of histones in vitro followed by ESI-MS/MS analyses identified the unique Cys110 residue within H3, exposed only in the open chromatin of transcriptionally active genes, as a K3 arylation target. These findings delineate new pathways associated with K3 induced stress and suggest a potentially novel role for H3 Cys110 as a nuclear stress sensor.
MOL(13474)

Introduction

Quinones encompass a broad array of biologically active compounds found both endogenously, as in the mitochondrial electron transporter ubiquinone, and exogenously where studies have documented their diverse health risks as carcinogenic, immunotoxic and cytotoxic agents (Bolton et al., 2000). As xenobiotic agents, quinones can disrupt cellular function via two distinct chemical pathways. Redox cycling quinones promote the generation of reactive oxygen species (ROS). Alternatively, as Michael acceptors, quinones covalently modify cellular nucleophiles, most prominently sulfur nucleophiles such as cysteine residues on proteins, creating potentially damaging arylation adducts. For quinones with both redox cycling and arylating potential, the contribution of each of these pathways to cellular stress response is difficult to fully quantify (Schneider et al., 2003; Abdelmohsem et al., 2003).

The vitamin K analog menadione (K3) is capable of both redox cycling and arylation and thus serves as a model bifunctional quinone. Previous studies have demonstrated that K3 stimulates phosphorylation of the mitogen activated protein kinase/extracellular regulated kinase (MAPK/Erk) pathway predominantly by arylation of key catalytic cysteine residues within protein tyrosine phosphatases (Abdelmohsem et al., 2003; Klotz et al., 2002; Osada et al., 2001). While it is generally the case that this pathway serves to stimulate cell growth and survival, recent findings have implicated non-survival aspects to excessive MAPK activation (Osada et al., 2002; de Bernardo et al., 2004; Choi et al., 2004; Dong et al., 2004; Tikoo et al., 2001), including induction of a non-apoptotic form of cell death known as oncosis (Tikoo et al., 2001; Romashko et al., 2003; Van Cruchte and Van Den Broeck, 2002; Trump et al., 1997). Thus, identifying early molecular targets of K3 induced cell stress will improve our understanding of quinone effects on cell function and fate.

The human breast cancer cells used in this study express abundant keratin 8 (k-8) and keratin 18 (k-18) as major components of their cytoskeletal structure. Keratins, limited in expression to tissues of epithelial origin, comprise the largest group of intermediate filament proteins and undergo extensive reorganization and modification during such cellular responses as mitosis, apoptosis and stress (Liao et al., 1997). While the functional consequences of keratin reorganization and post-translational modification remain poorly
understood, some insights have emerged (Liao et al., 1997; Schutte et al., 2004; He et al., 2002). Previous studies have documented that the stress activated protein kinases c-Jun N-terminal kinase (JNK) and p38 kinase target serine (Ser)-73 of k-8 for phosphorylation in response to a variety of cellular stresses and apoptosis inducing signals, while caspase cleavage of k-18 occurs as an early event during apoptosis thought to facilitate cellular breakdown (Schutte et al., 2004; He et al., 2002; Ku et al., 2002).

In this report, k-8 expressing breast cancer cells were studied following acute K3 stress with the aim of uncovering biological targets mediating the cytotoxicity induced by this redox active and arylating quinone. K3 exposure was found to initiate rapid MEK1/2 dependent Ser73 phosphorylation of k-8 which was maintained at a steady state level as cells progressively assumed the hallmark features of oncosis. Almost concurrent with k-8 phosphorylation was the appearance of K3 induced cellular fluorescence which was primarily nuclear in origin and suggested by fluorimetry to reflect K3 thiol arylation. Evidence is presented implicating histone H3 as a prominent K3 arylation target.
Materials and Methods

Reagents. Antibodies used in this study included the k-8 pSer73 specific mouse monoclonal Ab-7 (clone LJ4), the mouse monoclonal Ab-4 specific for k-8 (clone TS1), and the mouse monoclonal Ab-8 (clone L2A1) specific for k-18; all were obtained from Lab Vision. Total Erk1/2 and pErk1/2 specific rabbit polyclonal antibodies were from Cell Signaling. Phosphoserine antibodies were from Abcam. Horseradish peroxidase (HRP)-coupled goat anti-mouse and HRP-coupled goat anti-rabbit antibodies were from BioRad. Alexa Fluor 488 goat anti-mouse was from Molecular Probes. U0126, LY294002, DMNQ and SB203580 were from Calbiochem. SP600125 was from A. G. Scientific. Menadione (K3), p-benzoquinone (BQ), histones, cysteine, N-acetyl cysteine (NAC), glutathione, iodoacetic acid (IAA) and trypan blue were all obtained from Sigma.

Cell lines, treatment conditions, and cell viability assessment. Human breast cancer cell lines MCF7, T47D and SKBr3 were obtained from the American Type Culture Collection (ATCC). MCF7 cells were maintained in Dulbecco’s Modification of Eagle’s Medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro), 1% penicillin/streptomycin (Cellgro) and 10 ug/ml insulin (Sigma). T47D cells were maintained in RPMI (Cellgro) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 10 ug/ml insulin. SKBr3 cells were maintained in McCoys (Cellgro) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Treatment conditions for all cell lines involved plating ~1x10^6 cells in normal media onto 10 cm dishes, attachment and growth for 24 h followed by a change to estrogen-free culture conditions (phenol red-free DME H-16 supplemented with 10% charcoal stripped serum, 1% penicillin/streptomycin and 10 ug/ml insulin) for an additional 24 h to eliminate any potential estrogen stimulatory influences or estrogen metabolism to catechols and quinones. Cells were then treated as indicated before fractionation and extract preparation. Cell viability was assessed at timed intervals following initiation of culture treatment using twelve-well culture dishes containing ~40 x 10^3 cells per well. Replica plated for each treatment condition, wells were stained with Trypan Blue and cells were scored for dye exclusion.
counting at least 1500 cells per well from representative areas. Separate experiments assessing cell viability were repeated at least three times.

**Immunofluorescence and microscopy.** Cells were plated, grown and treated as described above on Lab-Tek II Chamber Slides (Nalge Nunc). To image k-8 by immunofluorescence, cells were permeabilized with 0.5% trition X-100 (Sigma), fixed with 4% paraformaldehyde (Sigma), and blocked with 5% normal goat serum (Rockland Biochemicals) for 1 h in wash buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1% BSA). Fixed, permeabilized and blocked cells were first incubated with the k-8 (Ab-4) or the k-8 pSer73 antibody (Ab-7) at 1:300 dilution for 1 h at room temperature in wash buffer containing 2.5% goat serum, and then incubated for 1 h at room temperature with a fluorescent goat anti-mouse secondary (Alexa Fluor 488, Molecular Probes) in wash buffer containing 2.5% goat serum. Nuclear DNA was imaged by DAPI staining (0.5 ug/ml), and slides were mounted (Vector Laboratories), viewed and photographed using a Nikon E800 upright fluorescence microscope equipped with filter cubes: (Ex 460-500 DM 505 BA 510-560), (Ex 530/50 DM 565 BA 610/75) and (Ex 360/40 DM 400 BA 460/50). For immunofluorescence studies not employing primary or secondary antibodies, treated and control cells grown in Lab-Tek II Chamber Slides (Nalge Nunc) were permeabilized with 0.5% trition X-100 (Sigma) in PBS, fixed with 4% paraformaldehyde (Sigma) then washed extensively with multiple PBS changes. DNA was visualized by addition of DAPI (0.5 ug/ml) except in those experiments where its omission was noted and slides were mounted, viewed and photographed as described above.

**Cell fractionation, nuclear extracts, and genomic DNA analysis.** Following aspiration of culture media and one ice cold PBS wash, cells growing on 10 cm dishes were harvested using a cell scraper and 0.7 ml of ice cold cell lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 10 mM NaFl, 5% glycerol, 0.45% NP-40 and Roche mini-complete protease inhibitor cocktail). Nuclei with insoluble cytoplasmic components were pelleted at 4 °C in a microcentrifuge for 4 min at 3,000xg. The supernatant
MOL(13474)

constituted the soluble cytosol, while the pelleted material was resuspended in 90 ul of DNase I digestion buffer (20 mM Tris pH 7.5, 100 nM NaCl and 10 mM MgCl₂) and incubated with 300 units of DNase I at room temperature for 5 min. Following DNase I digestion, complete solubilization of the pelleted material was achieved by addition of SDS (to 1%) to the DNase I reaction. Acid extraction of the pelleted fraction was performed with 0.2 M HCl. Aliquots of the acid extracted material were treated with 1 mM K₃ for 1 h at 25 °C and then neutralized with 0.5 Tris pH 11 before protein gel electrophoresis. Genomic DNA was extracted and analyzed for DNA laddering as follows. Control and treated cells were harvested in buffer (20 mM Tris pH 7.5, 50 mM EDTA, 100 mM NaCl, 0.5% SDS and 200 ug/ml Proteinase K), incubated at 56 °C for 5 h, phenol/chloroform extracted, ethanol precipitated and resuspended in TE with the addition of RNase A to remove background RNA. The resulting genomic DNA was electrophoresed on a 1% agarose gel using 1xTBE. As a positive control for DNA laddering, genomic DNA was prepared from cultured SKBr3 induced into apoptosis by 6 h treatment with conditioned media from cultures grown 5 d beyond confluence.

**Western analyses.** Equal amounts of protein from the various treatments were mixed with 2X SDS sample buffer (125 mM Tris pH 6.8, 20% glycerol, 2% SDS, 0.28 M 2-mercaptoethanol and 0.5% Bromphenol Blue), heated for 5 min at 90 °C and electrophoresed on NuPAGE 4-12% Bis-Tris gels (Invitrogen) using NuPAGE MOPS SDS running buffer (Invitrogen) and full range Rainbow recombinant protein molecular weight markers (Amersham Pharmacia). Gels were electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia) in standard transfer buffer (25 mM Tris, 200 mM glycine with 20% methanol) at 250 mA for 1 h at room temperature. Membranes were then blocked for 30 min in blocking buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.3% Tween-20 and 4% Nonfat dry milk powder by weight). Following blocking, membranes were incubated overnight at 4 °C with the primary antibody in blocking buffer using a 1:1000 dilution of the supplied antibody concentration. Membranes were then washed three times for 5 min each in blocking buffer without the milk powder, incubated with a HRP conjugated secondary antibody at 1:10,000 dilution in blocking buffer for 1 h at room temperature, and washed three
Membranes were then developed using SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer’s instructions.

**Fluorimetry and fluorescence illumination of gel-separated proteins.** K3 stock (0.1M in DMSO) was diluted to 1mM in either water or a 50:50 ethanol:water mixture for fluorimetry using a Pekin Elmer Luminescence Spectrometer model LS50B. To induce K3 thioether formation, K3 from stock solution was added directly to 100 mM cysteine or 100 nM glutathione solutions in water to a final 1mM K3 concentration. After allowing the reaction to proceed for 10 min at room temperature, the mixture was analyzed in the spectrometer at the indicated excitation and emission wavelengths. For analysis of cells lysates, 100 ul of nuclear lysate (standardized for equal protein content) treated with vehicle or K3 stock was diluted after treatment with 400 ul water and analyzed in the spectrometer. For fluorescence detection of gel separated proteins, extracts prepared as described above were gel separated as for Western analyses, washed extensively in water following electrophoresis, imaged using a UVP Epi ChemiII Darkroom UV transilluminator, and photographed using a SYBR Green filter (515nm-570nm) with fully opened aperture and a 7 sec exposure time.

**Histone derivatization and mass spectrometry**

A stock solution of commercially acquired human histones (Sigma; 10 ug/ul) was prepared in water, and a 2 ul aliquot of the stock solution was either untreated (control) or treated with K3 to a final concentration of 3 mM in an acid environment (0.5% acetic acid v/v) for 1 h at 37°C. After a 2 fold dilution with ammonium bicarbonate (pH 8, 25 mM), the control and K3 treated samples were treated with dithiothreitol (DTT, 4 mM) to reduce any disulfide bonds followed by addition of iodoacetic acid (6 mM) for alkylation of cystene thiols (control) or alkylation of any remaining cysteine thiols that were not arylated by the initial K3 treatment. The samples were then diluted 10 fold with ammonium bicarbonate followed by digestion with trypsin (20 ng/ul) and then with a second endoproteinase, Asp-N (40 ng/ul). Mixtures of
derivatized peptides were desalted and concentrated using C-18 ziptips (Millipore, Bedford, MA USA) for subsequent mass spectrometric analysis. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed using a QSTAR Pulsar mass spectrometer (MDS Sciex, Concord, Ontario Canada) operated in the positive-ion mode with a nano-electrospray needle voltage of 2300V. Loading and elution of peptides onto the LC column was performed using a binary solvent system: solvent A (0.05% formic acid in 98% H2O/ 2% AcN) and B (0.05% formic acid in 2% H2O/ 98% acetonitrile) at a flow rate of 300 nl/min and a gradient of 2% solvent B (from 0-5 min), and then 2-70% solvent B (from 5-55 min). Data (MS and tandem MS) were recorded continuously; and peak lists for database searching were created using a script from within Analyst software, and peptide sequences were identified using an in-house version of the database search package, Protein Prospector (UCSF). Database searches were performed by pre-selecting K3 or IAA modified amino acid residues of cysteine and/or lysine incorporated in the Protein Prospector software.
Results

K3 rapidly induces k-8 Ser73 phosphorylation. Previous studies had documented the activation of MAPK (Erk1/2, p38, JNK) and PI3K/Akt signaling in response to quinone exposure (Klotz et al., 2002; Osada et al., 2002; Seantor et al., 2003). Using phosphoserine specific antibodies to screen for proteins serine phosphorylated following K3 treatment, an intensely reactive band at 52 kDa was detected and subsequently identified by MS to be k-8. To further explore this response and eliminate potentially confounding effects from exogenous estrogens or endogenous quinone metabolites of estrogen, estrogen receptor (ER)-positive MCF7 cells were cultured in phenol-free media supplemented with charcoal-stripped serum for 24 h prior to 30 min K3 (100 uM) treatment. Parallel cultures were treated with an equimolar dose of the structurally similar redox cycling and non-arylating quinone, DMNQ. Harvested monolayer cells were lysed in hypotonic buffer and separated into two components, a soluble cytoplasmic fraction (Cyto) and a pellet fraction (Nu) containing intact nuclei and insoluble keratins filaments. As shown in Fig. 1B, the majority of k-8, despite being a cytoplasmic protein, pelleted predominantly as insoluble filaments with only a small soluble pool remaining in the cytoplasmic fraction (Chou et al., 1993). Using monoclonal antibodies against k-8 and k-8 pSer73, Fig. 1A shows that K3 significantly increased k-8 pSer73 above control levels while DMNQ treatment produced only a small increase in k-8 pSer73. Comparison of the Cyto and Nu fractions showed that the majority of k-8 pSer73 was present in the insoluble filamentous Nu fraction, as shown in Fig. 1B. The localization of Erk1/2 primarily within the Cyto fraction confirmed the efficiency of the separation protocol (Fig. 1B). Titration of K3 concentrations demonstrated a sharp decline in k-8 pSer73 formation below 50 uM, with <10 uM K3 producing little of no k-8 pSer73 relative to K3 doses ≥50 uM, as shown in Fig. 1C. Also, near maximal k-8 pSer73 levels occurred within 0.5 h of K3 exposure and remained unabated even after 6 h treatment (Fig. 1D). In two other human breast cancer cell lines, ER-negative SKBr3 and ER-positive T47D, 100 uM K3 induced k-8 pSer73 formation in a manner similar to that seen with MCF7 (data not shown).
To examine potential changes in MCF7 cytoskeletal architecture induced by K3 treatment, total k-8 and k-8 pSer73 were imaged by fluorescence microscopy. As shown in Fig. 2, k-8 containing cytoplasmic filaments were evident before treatment and remained relatively unchanged even 3 h after K3 (100 uM) treatment. In contrast, strong induction of k-8 pSer73 was seen in well defined cytoplasmic filaments within 3 h of K3 treatment, and there was minimal k-8 pSer73 apparent in untreated MCF7 cells.

MEK1/2 inhibition prevents K3 induced k-8 pSer73 and delays the onset of K3 cytotoxicity. To determine which signaling pathway(s) mediate K3 induction of k-8 pSer73, human breast cancer cells were pretreated for 10 min with inhibitors of MEK1/2 (10 uM U0126), p38 (10 uM SB203580), JNK (30 uM SP600125) or PI3K/Akt (10 uM LY20889) before the addition of K3 (100 uM x 30 min). As shown in Fig. 3A, the MEK1/2 inhibitor reduced k-8 pSer73 phosphorylation to control levels while the p38, JNK and PI3K/Akt inhibitors had no impact on K3 induced k-8 pSer73. Also shown in Fig. 3A was the suppression of K3 induced k-8 pSer73 resulting from concurrent treatment of cells with the thiol donor and arylation quencher N-acetyl cysteine (NAC), a result consistent with previous observations (Abdelmohsen et al., 2003). Confirmation that the immediate downstream targets of MEK1/2 signaling, Erk1/2, were phosphorylated by K3 induced MEK1/2 activation and that this activation was inhibited by U0126 pretreatment is shown Fig 3B. Additionally, Fig. 3 confirms the inhibition by NAC of K3 induced MEK1/2 activation. DMNQ, which produced a slight increase in k-8 pSer73 (Fig. 1A), induced activation of Erk1/2 though not to the degree elicited by K3 (Fig. 3B).

Since activation of the MAPK pathway and k-8 pSer73 have been associated with cellular stress and U0126 prevented K3 induced Erk1/2 and k-8 phosphorylation, the ability of U0126 (10 uM) to prevent K3 (100 uM) induced cytotoxicity was examined by measuring MCF7 cell viability at hourly intervals following K3 treatment. As shown in Fig. 3C, inhibition of Erk1/2 and k-8 phosphorylation extended cell viability by as much as 25% after 3 to 4 h of K3 treatment relative to cells not receiving U0126 co-treatment; however, by 6 h after K3 treatment there was virtually complete loss of MCF7 cell viability regardless of U0126 co-
treatment. The morphological features of all breast cancer cell lines examined (MCF7, SKBr3, T47D) showed comparable loss of cell viability within 6 h of K3 treatment without evidence of any classical features of apoptosis such as membrane blebbing, cell shrinkage, chromatin condensation or DNA fragmentation. Adherent but non-viable K3 treated cells all retained their cytoplasmic k-8 filamentous structures, nuclear contours and normal appearing DAPI stained chromatin (shown in Fig. 2); and upon examination of their genomic DNA treated cells showed some evidence of DNA degradation but no evidence of DNA laddering signifying apoptosis (Fig. 3D). Observed light microscopic changes in cell morphology associated with K3 cytotoxicity were most consistent with oncosis (Van Cruchte and Van Den Broeck, 2002; Trump et al., 1997), the cell death pathway induced by various physical and chemical stresses and characterized by swelling of intracellular organelles, including mitochondria and nucleoli, and development of a refractive fringe around cell margins due to loss of plasma membrane integrity and cell junctions.

**Rapid induction of nuclear fluorescence consistent with K3 thiol arylation.** Fluorescence microscopy revealed that K3 treated breast cancer cells rapidly became fluorescent, most prominently within the nucleus, while no comparable fluorescence was evident in vehicle treated control cells. While the capacity of K3 to modify thiol groups through arylation has been well documented (Topopeus et al., 1993; Feng et al., 1999; MacDonald et al., 2004), there are no reports that we are aware of describing K3 induced cellular fluorescence. Following detergent (0.5% triton) permeabilization to remove soluble cell components and fixation in paraformaldehyde (4%), K3 treated (100 uM) MCF7 cells acquired diffusely intense nuclear fluorescence when excited at 480nm and imaged at 535nm, or when excited at 535nm and imaged at 640nm (Fig. 4). Of note, the K3 induced nuclear fluorescence viewed over 0.3 seconds left the k-8 immunofluorescence imaged over 0.07 seconds essentially undistorted (Fig. 2). This nuclear fluorescence appeared to increase in a relatively linear manner following K3 administration, with emission intensity at 3 h appearing 3 to 4-fold greater than that seen after 0.5 h treatment, and exceeding control autofluorescence by more than an order of magnitude (Fig. 4). The linear time course of K3 induced nuclear fluorescence which
emerged over several hours contrasts with the kinetics of k-8 pSer73 formation which reached maximal steady-state levels within 1 h (Fig. 1D). That protein arylation by K3 accounted for this nuclear fluorescence is supported by three observations. Firstly, detergent permeabilization followed by paraformaldehyde fixation allowed for the elimination of soluble factors including unreacted K3 and soluble K3 complexes. Secondly, treatment with DMNQ which is incapable of arylation produced no cellular florescence above untreated cell autofluorescence (data not shown). Thirdly, both K3 induced k-8 phosphorylation and K3 induced nuclear fluorescence were reduced to control levels by concurrent cell treatment with the thiol donor NAC, as shown in Fig. 3A and Fig 4 respectively.

To examine the excitation and emission properties of fluorescence induced by various K3 arylated products, in vitro fluorimetry studies were preformed using cysteine and glutathione as soluble thiol donors. Unreacted K3 exhibited minimal fluorescence with best results attained with 340nm excitation which produced a poorly defined emission peak centered at 420nm (Figs. 5A and 5B). In contrast, following the virtually instantaneous reactions (at room temperature) between K3 and either cysteine or glutathione, strong K3 thioether emission peaks at 480nm with 340nm excitation and at 420 nm with 360nm excitation were observed for cysteine and gluathione respectively (Fig. 5A, B). This >10-fold enhancement in K3 thioether fluorescence over unreacted K3 was observed in both water and 50:50 water:ethanol vehicle, assuring that the low aqueous solubility of K3 was not biasing the fluorimetry results (data not shown). No fluorescence was detected from cysteine or glutathione without addition of K3. In near agreement with the K3-glutathione thioether fluorescence properties, a nuclear protein extract from cells treated with K3 (100 uM x 3h) produced a strong fluorescence emission peak at 390nm relative to untreated control extract with 340nm excitation (Fig. 5C). These vitro studies demonstrate that K3 experiences a >10 fold enhancement in fluorescent activity upon thioether formation, an observation which strengthens the conjecture that the K3 induced nuclear fluorescence observed in Fig. 4 results from K3 thioether formation. To simulate in a cellular environment these in vitro studies which demonstrated excitation of the K3 thioether around 360nm, monolayer MCF7 cells were treated with K3 for 3h and then prepared similarly to those shown in Fig. 4 except that staining with DAPI, which
MOL(13474)

absorbs at 360nm, was eliminated. The cells were then analyzed by fluorescence microscopy using 360 (+/- 20) nm excitation and imaged at 460 (+/-25) nm for 0.3 s (Fig. 5D). While no fluorescence was detected in untreated cells, K3 treated cells demonstrated a diffuse and predominantly nuclear fluorescence pattern similar to that shown in Fig. 4.

**Histone H3 is the major nuclear target of K3 thiol arylation.** To identify protein targets of K3 arylation, nuclear extracts from K3 treated cells were resolved on protein gels and examined for fluorescent bands. Following electrophoretic separation of control and K3 treated MCF7 extracts, polyacrylamide gels were thoroughly washed with water then imaged using a wide spectrum UV transilluminator coupled to a SYBR green filter (515-570 nm). Coomassie staining was subsequently performed to establish equal protein loading and molecular weight sizes. As shown in Fig. 6, strongly UV illuminated protein bands were apparent in the K3 treated extracts with only faintly detected bands apparent in the untreated control nuclear extracts. Careful alignment of the UV transilluminated image with the Coomassie stained image revealed prominent K3 induced bands at 47 kDa, 43 kDa, and 17 kDa (Fig. 6). The intensely staining Coomassie bands at 52 kDa, corresponding to k-8, and 48 kDa, verified as k-18 by antibody (data not shown), were not accompanied by any detectable bands under UV transillumination. Of the K3 induced UV transilluminated bands, only the 17 kDa species precisely aligned with a strong Coomassie band. As nuclear proteins of this molecular size and abundance are predominantly histones, antibodies were used to confirm that the strong Coomassie bands at 11 kDa, 15 kDa and 17 kD corresponded to histones H4, H2a/H2b and H3.3/H3, respectively (data not shown). Since H3 and its variants are the only histones that contain any cysteine residues (Cys110), the observed UV transilluminated protein within the 17 kDa band was most likely attributable to K3 induced thiol arylation of H3.3/H3. Supporting evidence for this proposal was obtained from in vitro K3 (1 mM) treatment of an acid extract of MCF7 nuclear pelleted material. As shown in Fig. 6, comparison of the in vivo and in vitro K3 treated nuclear proteins with their untreated controls showed similar Coomassie banding across all lanes while UV transillumination revealed the 17 kDa H3.3/H3 band most prominently only in the K3 treated lanes.
Histone H3 and its variant H3.3, which preferentially associates with transcriptionally active chromatin (McKittrick et al., 2004) are structurally identical except for four neutral amino acid differences within a short (10 amino acid) region adjacent to the conserved core domain containing Cys110 (Fig. 7A). Typically buried in the interior of the nucleosome, this C-terminal Cys110 for both H3.3/H3 only becomes chemically accessible and reactive during active gene transcription or other situations promoting open chromatin configuration (Sun et al., 2002; Bazett-Jones et al., 1996; Arents et al., 1991). Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) was used to confirm the ability of K3 to arylate H3.3/H3 at the thiol of Cys110. Following iodoacetic acid (IAA) treatment of control and K3 treated histones from a commercial preparation, sequential protease digestion by trypsin and AspN was used to release the fragment 106-DTNLCAIHAK-115 from H3.3/H3. K3 quinone modification to Cys110 would be expected to increase the molecular weight of 106-115 peptide from 1,084.5 amu to 1,254.5 amu if arylated with K3. An electrophilic attack of a quinone like K3 on a thiol moiety initially results in the reduction of the quinone to a dihydroquinone, but under aerobic conditions this dihydroquinone is rapidly oxidized partially or completely back to the quinone. Additionally, IAA modification of Cys110 in the control sample as well as to those Cys110 not modified by K3 in the K3 treated sample is expected to produce an IAA modified 106-115 peptide of molecular weight of 1142.5 amu. As shown in Fig. 7B panels I and III, for control and K3 treated samples respectively, ESI-MS/MS identified a well-defined peak at 1,142.5 amu (381.85^+3 m/z), corresponding to the anticipated mass of the IAA Cys110 modified 106-115 H3/H3.3 peptide fragment. However, as shown in Fig. 7B panels II and IV, for control and K3 treated samples respectively (note that the scale factor for panel II is reduced approximately 100-fold relative to panel IV), ESI-MS/MS detected a well-defined peak at 1254.5 amu (419.17^+3 m/z) corresponding to the presence of a Cys110 K3 modified 106-115 peptide only in the K3 treated histone sample. To precisely confirm the expected chemical alterations occurring on Cys110, selection and fragmentation of the IAA modified 381.85^+3 m/z ion and the K3 modified 419.17^+3 ion by MS/MS yielded multiple y (C-terminal) and b (N-terminal) ion series as shown in Figs. 8A and 8B respectively. The 161 amu difference between the y_6 (700.3 m/z) and y_5 (539.3 m/z) ions shown in...
Fig. 8A reflects a single IAA moiety coupled to the Cys110 residue (103 Da) while the 273 amu difference between the y₆ (812.3 m/z) and y₅ (539.3 m/z) ions shown in Fig. 8B reflects a single K₃ moiety coupled to Cys110. A similar ESI-MS/MS approach was attempted to detect K₃ arylated H₃.₃/H₃ from a digest performed on the 17 kDa protein band excised from gel separated nuclear extract of in vivo treated K₃ MCF7 cells. While ESI-MS/MS analysis produced 80% sequence coverage of H₃.₃/H₃, including successful detection of the Cys110 containing fragment (data not shown), K₃ modified Cys110 was not detected, probably reflecting the relatively low abundance of K₃ modified to unmodified H₃.₃/H₃. Also, as the 22-amino acid peptide fragment containing the four amino acids that distinguish H₃ from H₃.₃ (Fig. 7A) was not detected, any Cys96 modifications occurring to H₃ would have escaped identification.
Discussion

While previous studies have shown activation of the MAPK pathway by both redox cycling and arylating quinones (Abdelmohsem et al., 2003; Klotz et al., 2002; Osada et al., 2001; Seanor et al., 2003), the spectrum of quinone-targeted intracellular substrates remains largely uncharacterized. Results presented here demonstrate in human breast cancer cells (MCF7, T47D, SKBr3) that aberrant activation of MEK1/2 by the model quinone and vitamin K analog, K3, leads to sustained Ser73 phosphorylation of k-8, a primary intermediate filament constituent of all simple epithelial cells that heterodimerizes with its obligate cytokeratin partner, k-18. In conjunction with this cytoplasmic event, we demonstrated that K3 induces nuclear fluorescence resulting from its arylating capacity and in vitro evidence identified Cys110 of histone H3/H3.3 as a potential target of K3 thiol arylation.

Site-specific phosphorylation is known to influence both k-8 organization and dynamics as well as to modulate k-8 interacting proteins (Liao et al 1997; Schutte et al., 2004; He et al., 2002; Ku et al., 2002). Moreover, k-8 pSer73 formation in response to Fas receptor stimulation has been attributed to JNK activation (He et al., 2002). As shown here (Figs. 1-3), culture treatment of human breast cancer cells by K3 produced a rapid and sustained increase in k-8 pSer73 that was not mediated by JNK, p38 or P13K/Akt pathways as it was unaffected by their specific inhibitors: SP600125, SB203580 and LY20889, respectively. In contrast, K3 induced k-8 pSer73 was completely prevented by U0126, a specific inhibitor of MEK1/2, indicating that in MCF7, T47D and SKBr3 cells it is the MEK/Erk pathway that mediates Ser73 phosphorylation of k-8 by the redox active and arylating quinone, K3. Of note, culture treatment with the redox active but nonarylating quinone, DMNQ, produced only a marginal increase in intracellular k-8 pSer73, indicating that the arylating capability of K3 is largely responsible for its MEK/Erk-dependent phosphorylating effects on k-8. Furthermore, cotreatment of cultures with excess NAC completely prevented K3 induced phosphorylation of Erk1/2 and k-8. These observations are most consistent with the presence of a conserved redox sensitive
cysteine residue within the enzymatic pocket of most signal inhibiting phosphatases that is also very sensitive to quinone induced arylation (Abdelmohsem et al., 2003; Klotz et al., 2002).

While activation of the Erk1/2 pathway by growth factors and other mitogens is often associated with cellular growth and survival, its aberrant or sustained activation has also been linked with cell death (Osada et al., 2001; de Bernardo et al., 2004; Choi et al., 2004; Dong et al., 2004; Tikoo et al., 2001; Romashko et al., 2003). Indeed, complete inhibition of Erk1/2 and k-8 phosphorylation by U0126 delayed the onset but did not ultimately prevent the resultant cytotoxicity produced by K3 (Fig. 3C). In contrast, cotreatment with excess NAC effectively prevented K3 induced cytotoxicity, suggesting that arylation of intracellular targets apart from those involved in MAPK signaling predominantly determine K3 induced cytotoxicity. Morphologic and molecular characterization of K3 treated breast cancer cells during the 7 hours leading to their complete loss of viability was consistent with a form of cell death known as oncosis, typically seen in response to toxic injury and with hallmarks that include generalized organelle swelling and loss of plasma membrane integrity (Van Cruchte and Van Den Browck, 2002; Trump et al., 1997). Structurally intact k-8 filaments, relatively little genomic degradation with no DNA laddering, and an undistorted chromatin pattern on DAPI staining (Figs. 2, 3D, 4) even after 3 h of K3 treatment all pointed to an oncotic rather than apoptotic cell death process.

A novel result presented here was the demonstration that some cellular targets of K3 arylation, perhaps those more closely associated with its cytotoxic consequences, were readily visualized by fluorescence microscopy. While the fluorescent properties of quinones are widely appreciated, we have found no reports describing induction of fluorescence upon the reaction of biologically relevant quinones like K3 with intracellular substrates. As is the case for a number of other thiol modifying reagents, K3 thioether formation was shown to be associated with acquisition of enhanced K3 fluorescence where excitation at wavelengths between 340-360nm resulted in emission optima at wavelengths between 390-480nm, depending on the chemical nature of the K3 arylated substrates (cysteine, glutathione, nuclear extract; Fig. 5). The K3 fluorescence properties illustrated by the in vitro fluorimetry measurements shown here may not fully
MOL(13474)

represent all K3 reactions occurring in vivo; however, the concordance of these in vitro measurements with nuclear fluorescence imaged with 360nm excitation after K3 treatment of cultured breast cancer cells suggests that this in vivo fluorescence induction was a result of K3 thiol arylation. Cell cultures cotreated with U0126 showed no reduction in K3 induced nuclear fluorescence, whereas those cotreated with excess NAC showed complete inhibition of K3 induced fluorescence, indicating that this in vivo fluorescence was dependent on K3 arylation but was independent of its arylating induction of MAPK signaling. Curiously, studies with another well studied arylating agent, p-benzoquinone (BQ), demonstrated similar in vitro fluorescence induction on reaction with cysteine or glutathione substrates but no in vivo cellular fluorescence following BQ treatment of cultured cells, suggesting different in vivo permeability and/or chemical reactivity properties between BQ and K3 (data not shown). These apparent differences between the in vivo arylation properties of BQ and K3 could be further investigated using scanning electrochemical microscopy, since a recent study employed this approach to detect and monitor intracellular formation and efflux of the arylation product (thiodione) between K3 and glutathione in cultured hepatocytes (Mauzeroll et al., 2004).

Posttranslational modifications to histones are crucial to the regulation and organization of chromatin structure, as has been extensively documented and with more recent interest given to the use of mass spectrometry to delineate the full spectrum of histone modifications (Jenuwein and Allis, 2001; Berger, 2002; Syka et al., 2004). Histone H3 was implicated as a major target of intracellular K3 arylation from three initial observations: i) K3 induced cellular fluorescence was observed to be predominantly nuclear; ii) in vitro fluorimetry of nuclear extracts showed enhanced fluorescence following K3 treatment; and iii) gel separated proteins from K3 treated fluorescent nuclei revealed an abundant, UV transilluminated 17 kDa band aligning precisely with a Coomassie band confirmed by antibody and MS to be histone H3. As the only cysteine containing histone species, H3 and its variants all possess a core Cys110 residue that is evolutionarily conserved among metazoans (Sullivan et al., 2002). Some H3 variants, including the human H3.3 variants, contain an additional nearby cysteine residue (Sullivan et al., 2002). Functional and structural studies have demonstrated that the H3 core is buried within the nucleosomes when chromatin is in a closed configuration,
protecting Cys110 from chemical modification (Sun et al., 2002; Bazett-Jones et al., 1996; Arents et al., 1991). When chromatin is in an open configuration, which is characteristic of actively transcribing genes, Cys110 is readily accessible for thiol adduction (Sun et al., 2002; Bazett-Jones et al., 1996). Employing ESI-MS/MS, in vitro studies confirmed that K3 reacts readily and specifically with H3.3/H3, resulting in thiol arylation of its core Cys110 residue.

Taken together, these findings suggest that K3 induced cytotoxicity reflects oncosis primarily caused by the arylation property of this bifunctional quinone. While k-8 pSer73 formation marked an early response to K3 treatment, K3 induced nuclear fluorescence coincided with the development of oncosis and the arylation of a 17 kDa nuclear protein identified as H3.3/H3. From recognition of the unique structural susceptibility of H3.3/H3 with its core Cys110 residue vulnerable to thiol arylation during active gene transcription, it may be speculated that H3.3/H3 also functions as a nuclear sensor of chemical stress.
Acknowledgements

We thank Dr. David Nichols and Danielle Crippen from the Buck Institute’s Morphology Core for their helpful advice and assistance.
MOL(13474)

References


Dong J, Everitt JI, Lau SS and Monks TJ (2004) Induction of ERK1/2 and histone H3 phosphorylation within the outer stripe of the outer medulla of the Eker rat by 2,3,4-tris-(glutathione-S-yl)hydroquinone. Toxicol. Sci. 80 350-357

MOL(13474)


MOL(13474)
Footnotes:

This work was supported by NIH-sponsored grants R01-CA71468, R01-CA36773, R01-AG020521; California Breast Cancer Research Program grant CBCRP 10YB-0125; NCRR-RR01614 facility grant support to the UCSF Mass Spectrometry Facility; and generous donations to the Buck Institute by the Oracle Corporate Giving Program and the Hazel P. Munroe Memorial.

Request for reprints: Dr. Christopher Benz

Program of Cancer and Developmental Therapeutics, Buck Institute for Age Research
8001 Redwood Blvd., Novato, CA 94945

tel #: 415-209-2092;
**Figure Legends**

**Figure 1.** Menadione (K3) induces Ser73 phosphorylation of keratin-8 (pSer73 k-8) in MCF7 cells. **A.** Western blots of MCF7 nuclear pellet fractions (also containing insoluble cytoplasmic keratin filaments) from vehicle treated control cultures (C) and cultures treated with 100uM K3 or DMNQ (DQ) for 30 min. As shown, immunoblots were probed with antibodies specific for total k-8 or pSer73 k-8. **B.** Western blots of nuclear pellets (Nu) or soluble cytosols (Cyto) from MCF7 cultures treated as in A and probed with antibodies for total k-8, pSer73 k-8, or Erk1/2 (42/44 kDa). **C.** Western blots of nuclear pellets from MCF7 cultures treated for 30 min with the indicated concentrations of K3 and probed as indicated. **D.** Western blots of nuclear pellets from MCF7 cultures treated with 100 uM K3 for the indicated times (h) and probed as shown.

**Figure 2.** Immunocytochemical detection of k-8 and pSer73 k-8. Control and 3 h K3 treated MCF7 cells were immunocytochemically examined by fluorescence microscopy for total k-8 (top panel) and pSer73 k-8 (bottom panel) expression. An exposure time of 0.06 seconds was used to obtain total k-8 and pSer73 k-8 fluorescence images. DAPI staining was used to distinguish MCF7 nuclei.

**Figure 3.** MAPK dependence of K3 effects on pSer73 k-8, pErk1/2, cell survival and DNA integrity. **A.** Western blots of the nuclear pellets from MCF7 cultures treated for 30 min with 100 uM K3 and the indicated agents (C = vehicle control; U = 10 uM U0126; SB = 10 uM SB203580; SP = 30 uM SP600125; LY = 10 uM LY20889; and N = 10 mM N-acetyl cysteine), and probed with antibodies as described in Fig 1. **B.** Western blots of nuclear pellets from MCF7 cultures treated for 30 min with 100 uM K3 (+/- cotreatment with 10 uM U0126), 100 uM K3 with 10 mM NAC (K/N), or 100 uM DMNQ (DQ), and then probed with antibodies to total or phosphorylated Erk1/2 (42/44 kDa). **C.** Survival of MCF7 cells from cultures treated for the
indicated times (h) with 100 uM K3 with or without 10 uM U0126, measured by trypan blue dye exclusion. Error bars represent standard error from three replica plated experiments. **D.** Ethidium stained DNA agarose gel of genomic DNA isolated from SKBr3 cells treated in culture for 3-24 h with 100 uM K3. As a positive control for SKBr3 apoptosis, cells were exposed for 6 h to an apoptosis-inducing conditioned media (Ap) to demonstrate induction of DNA laddering. HindIII digested lambda phage were used as DNA size markers (M).

**Figure 4.** Fluorescence microscopy of K3 treated MCF7 cells. Control, 0.75 h 100 uM K3 treated, 3 h 100 uM K3 treated, or 3 h 100 uM K3 and 10 mM NAC (K3/NAC). MCF7 cells were imaged by fluorescence microscopy using an exposure time of 0.30 seconds for detection with Ex480nm/Em535nm filtering (top panel) and an exposure time of 0.25 seconds for detection with Ex535nm/Em610nm filtering (middle panel). DAPI fluorescence was used to image MCF7 nuclei (bottom panel).

**Figure 5.** K3 fluorescence following K3 thioether formation simulates the nuclear fluorescence from K3 treated MCF7 cells. **A.** Emission spectra of 1 mM K3 (red line) in water or in the presence of 0.1 M aqueous cysteine solution (blue line), excited at 340nm. **B.** Emission spectra of 1 mM K3 (red line) in water or in the presence of 0.1 M aqueous glutathione (GST) solution (blue line), excited at 360nm. **C.** Emission spectra of solubilized nuclear pellet from MCF7 cell cultures treated with vehicle alone (Control Extract, red line) or for 3 h with 100 uM K3 (K3 Extract, blue line), excited at 340nm. **D.** Fluorescent microscopy of MCF7 cells treated for 3 h with 100 uM K3 and prepared as described in Fig. 4 except for the elimination of DAPI staining were imaged with Ex360/Em460nm filtering and an exposure time of 0.3 seconds. The image is shown using green false coloring.

**Figure 6.** K3 modified histone H3 detected by UV transillumination. Gel separated proteins from the nuclear pelleted material of control (C) or 3 h 100 uM K3 treated (K3) MCF7 cells (in vivo) or from the acid
extracted nuclear pelleted material from control MCF7 cells (in vitro) treated with 1 mM K3 (K3) or untreated (C) were photographed for 7 seconds using a UV transilluminator equipped with a SYBR Green (515nm-570nm) filter (left panel). Continued exposure of the gel to UV transillumination resulted in the gradual appearance of bands in both K3 and C lanes. Precise alignment of the Coomassie stained protein gel image (right panel) with UV transilluminated image identified the 17 KDa histone H3/H3.3 Coomassie band as aligning with a prominent UV stimulated band in K3 lanes. Molecular weight markers and known histone (H3, H2A/H2B, H4) and keratin (k-8, k-18) bands are indicated.

**Figure 7.** Mass spectrometry (MS) detection of K3 thiol arylated or carboxymethylated peptide within histone H3.3/H3.  

**A.** Primary structure of histones H3.3 and H3 in the region of interest showing the 4 amino acids distinguishing the two isoforms. Amino acids from 84-105 are shown in small type and were not detected by MS; amino acids detected by MS are shown in large type. The boxed peptide (amino acids from 106-115) contains the K3 or iodoacetic acid (IAA) modified Cys110 detectable after H3.3/H3 double digestion with trypsin and Asp-N.  

**B.** LC-ESI-MS data for the IAA treated negative controls (panels I and II) and the K3 treated histone conditions (panels III and IV). Panels I and III show the triply charged ion (381.85+3 m/z peak) eluted at 19.7 min and corresponding to the carboxymethylated 106-115 peptide resulting from IAA treatment. Panels II and IV show the signal for the analogous ion (419.18+3 m/z peak as well as its isotopic variants at 419.51 and 419.83, evident only in panel IV) eluted at 28.2 min and corresponding to the K3 adducted 106-115 peptide. In contrast to the prominent 419.18+3 m/z peak evident in panel IV, note the absence of any 419.18+3 m/z peak in panel II and the near 100-fold lower ordinate scale.

**Figure 8.** Tandem MS identification of Cys110 modifications in treated histone H3.3/H3. LC-ESI-MS/MS analysis of the carboxymethylated 381.85+3 m/z peak (Panel A) and the K3 adducted 419.17+3 m/z peak (Panel B) from double digests of the treated histone preparations described in Figure 7.  

**A.** Fragmentation of the 381.85+3 m/z peak showing the 161 amu difference between the y6 (700.3 m/z) and y5 (539.3 m/z) ions and
 confirming the presence of a single IAA moiety coupled to Cys110 (Cys-IAA). B. Fragmentation of the 419.17+3 m/z peak showing the 273 amu difference between the y₆ (812.3 m/z) and y₅ (539.3 m/z) ions and confirming the presence of a single K3 moiety coupled to Cys110 (Cys-K3). For both A and B data, b and y ions confirm the amino acid sequence, and the mass differences between y₅ and y₆ identify Cys110 as the modification site.
Figure 1

A. C K3 DQ
   pSer73 k-8
   total k-8

B. Nu  Cyto
     C  K3  C  K3
     pSer73 k-8
     total k-8
     Erk1/2

C. K3 (µM): 100 50 10 1 0
   pSer73 k-8
   total k-8

D. 100 µM K3 (h): 0 0.5 2 6
   pSer73 k-8
   total k-8
Figure 3

A. 

\[
\begin{array}{ccccccc}
 & K3 + \\
C & N & U & SB & SP & LY \\
pSer73 k-8 & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{total k-8} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\end{array}
\]

B. 

\[
\begin{array}{ccccccc}
 & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
C & K3/U & K3 & K3/N & DQ \\
pErk1/2 & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{total Erk1/2} & \text{---} & \text{---} & \text{---} & \text{---} \\
\end{array}
\]

C. 

\[
\begin{array}{cccccccc}
\text{Percent Survival} & 100 & 90 & 80 & 70 & 60 & 50 & 40 & 30 & 20 & 10 & 0 \\
\text{Hours After Treatment} & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\end{array}
\]

- K3 + U
- K3

D. 

\[
\begin{array}{cccccccc}
\text{M} & \text{Ap} & 0h & 3h & 7h & 24h \\
\end{array}
\]

\[\text{K3}\]
Figure 6

A.  

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.  

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 105 kD
- 75 kD
- k-8 (52 kD)
- k-18 (48 kD)
- 35 kD
- 25 kD
- H3.3/H3
- H2A/H2B
- H4
Figure 7

A. histone H3.3 -TDLR-fqsaigalqaceaylvglf-DTNLCAIHAKRVTI-

histone H3

B. (I) 381.85 382.19

m/z

(II) 419.0 420.0

(III) 381.83 382.17

(IV) 419.18 419.51
Fig 8

(A) Cys-IAA

(B) Cys-K3

DTNLC*AIHAK

y8 y7 y6 y5 y4 y3 y2 y1

b2 b3 b4

m/z

400 500 600 700 800 900 1000