Cancer Letters 332 (2013) 35–45

Original Articles

Cucurbitacin B potently suppresses non-small-cell lung cancer growth: Identification of intracellular thiols as critical targets

Hina Kausar a, Radha Munagala a, Shyam S. Bansal b, Farrukh Aqil a, Manicka V. Vadhanam a, Ramesh C. Gupta a,b,*

a James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202, United States
b Department of Pharmacology and Toxicology, University of Louisville, Louisville, KY 40202, United States

A R T I C L E   I N F O

Article history:
Received 31 October 2012
Accepted 4 January 2013

Keywords:
Cucurbitacin B
Lung cancer
Cellular thiols
Apoptosis

A B S T R A C T

Cucurbitacin B (CuB), has recently emerged as a potent anticancer agent; however, its efficacy in non-small-cell lung cancer (NSCLC) and the mechanism(s) initiating its biological effects remain largely unclear. In this study, CuB potently suppressed the growth of four NSCLC cells (H1299, A549, HCC-827 and H661) In vitro and the highly aggressive H1299 xenograft in vivo. CuB significantly altered the actin cytoskeletal assembly, induced G2/M cell-cycle arrest and mitochondrial apoptosis through the modulation of several key molecular targets mediating the aforementioned processes. Interestingly, all cellular effects of CuB were completely attenuated only by the thiol antioxidant N-acetylcysteine (NAC). Furthermore, pretreatment with glutathione synthesis inhibitor butithione-sulfoxime (BSO), significantly exacerbated CuB’s cytotoxic effects. To this end, cells treated with CuB revealed a rapid and significant decrease in the levels of protein thiols and GSH/GSSG ratio, suggesting disruption of cellular redox balance as the primary event in CuB’s cytotoxic arsenal. Using UV and FTICR mass spectrometry we also demonstrate for the first time a physical interaction of CuB with NAC and GSH in a cell-free system suggesting that CuB interacts with and modulates cellular thiols to mediate its anti-cancer effects. Collectively, our data sheds new light on the working mechanisms of CuB and demonstrate its therapeutic potential against NSCLC.

Cancer Letters 332 (2013) 35–45

1. Introduction

Despite recent advances in modern medicine, non-small cell lung cancer (NSCLC) still continues to be a radical health concern [4]. Dysregulation of several key signal transduction pathways, defects in cell cycle progression and resistance to apoptotic signaling has largely been attributed to the uncontrolled proliferation observed in lung tumor cells [16,34], thus warranting the need to characterize new agents that can effectively re-enforce the defense machinery of tumor cells propelling them to undergo apoptosis.

Over the recent years, apoptosis induction due to alterations in intracellular redox status by certain oxidative or non-oxidative stimuli has become a focus of extensive research [11]. Besides reactive oxygen species (ROS), intracellular thiols – GSH and the protein thiols are the two major determinants of cellular redox equilibrium, functioning as regulators of cell survival through their effects on gene activation, cell cycle arrest and apoptosis [14,26].

Hence, manipulation of cellular redox status represents a viable strategy for apoptosis induction by anti-cancer agents. Lately, the natural triterpenoids, cucurbitacins have become a subject of intense investigation because of their high efficacy to inhibit varied cancers [30,38]. Based on molecular structural differences, the cucurbitacins are classified into twelve groups (denoted A through T) [7]. Amongst them, the terpenoid cucurbitacin B (CuB) has recently gained increasing attention because of its potent in vitro and in vivo antitumor effects [8,42,45]. The biological effects of CuB has been shown to be mediated mainly through its effects on the actin cytoskeleton, intracellular ROS levels, cell-cycle arrest and apoptosis through the modulation of COX2, NF-kappaB, JAK2/STAT3, ERK and MAPK pathways, [18,21,22,42,45,48,50]. While many of these studies have mostly delineated events occurring relatively late after the exposure of cells to CuB, the mechanisms initiating CuB’s biological effects are still not clear yet. Also with the exception of a few published studies exploring the cytotoxic effects of CuB and CuI (another member of the cucurbitacin family) on lung cancer growth [20,21,27], detailed mechanistic evaluation of CuB’s antiproliferative effects in lung cancer is still lacking. The present study was therefore designed to investigate the anticancer potential of CuB against NSCLC and identify the underlying mechanisms initiating its cytotoxic effects.

* Corresponding author. Address: James Graham Brown Cancer Center, University of Louisville, 304E Delia Baxter II, 580 S. Preston St., Louisville, KY 40202, United States.
E-mail address: rgupta@louisville.edu (R.C. Gupta).
2. Materials and methods

2.1. Reagents and culture materials

CuB (95% pure) was purchased from Phytomyco Research (Greenville, NC), NAC and BSO (Sigma–Aldrich, St. Louis, MO), MTT (Calbiochem, Gibbstown, NJ), fluorescein-conjugate H_2DCFDA, cell culture medium and other supplements (Invitrogen, Grand Island, NY), were obtained from the sources cited.

2.2. Cells, culture conditions and treatments

H1299 (p53null/EGFRWT), H661 (p53mutant/EGFRWT), and A549 (p53WT/EGFRWT) cells were kindly gift from Drs. Paula Bates and Keith Davis (University of Louisville) and Dr. C.G. Gairola (University of Lexington KY) respectively. HCC-827 (p53WT/EGFR mutant) cells were obtained from ATCC. The cell lines were authenticated by short tandem repeat sequencing and mycoplasma detection analysis performed at the John Hopkins genetic resources core facility (Baltimore, MD). Cells were maintained in recommended medium at 37 °C in 5% CO_2 and humidified air. For all studies, cells at 70–75% confluency were treated with vehicle (0.1% DMSO) or indicated concentrations of CuB and 5 mM NAC each of, NAC and/or BSO.

2.3. Cell-viability assay

Cells (4.5 × 10^4/well) were cultured at 37 °C in 96-well plates with vehicle, varying concentrations of CuB (0.025–1 μM) for 24, 48 or 72 h, 5 mM NAC + CuB, or 5 mM BSO + CuB + 5 mM NAC for 72 h and cell viabilities were measured using the MTT reduction assay [24].

2.4. In vivo xenograft assay

Female athymic nude (nu/nu) mice (5–6-week old), from Harlan laboratories (Indianapolis, IN) were maintained in accordance with the Institutional Animal Care and Use Committee guidelines. A suspension of 1 × 10^6 H1299 cells in vehicle-free media were mixed with Matrigel matrix (Becton Dickinson, Bedford, MA) and subcutaneously injected into the left flank of each mouse. The following day, mice were randomly divided into 2 groups of 5 mice each and treated intraperitoneally with either vehicle (10% DMSO in PBS) or CuB (1 mg/kg body weight) on alternate days. Tumor size and animal weights were monitored weekly. After 5 weeks of treatment, the experiment was terminated due to severe scabbing of few tumors in the control group. Post-euthanasia, tumors were harvested and snap frozen in liquid nitrogen for further analysis.

2.5. Confocal laser scanning microscopy for F-actin staining

H1299 cells grown in 8-well chambered slides were treated with vehicle, CuB or the p38 inhibitor SB203580 (SelleckChem, Boston MA) for 2 h and then labeled with rhodamine–phalloidin as described previously [45]. Post-staining, the cells were observed within minutes of CuB treatment as evidenced by rapid alterations in H1299 cellular morphology and remodeling via p38MAPK/Hsp27 activation.

2.6. Intracellular ROS measurement

Early levels of intracellular ROS generation were assessed spectrofluorimetrically by oxidation of H_2DCFDA [1]. Briefly, cells seeded on black 96-well tissue culture plates were washed once with PBS, then incubated concomitantly with different concentrations of CuB and 100 μM H_2DCFDA in PBS. Fluorescence intensity was measured immediately after addition (0 min), then at 15, 45, 90 and 180 min at excitation and emission wavelengths of 490 nm and 535 nm, respectively.

2.7. Cell-cycle analysis

Flow cytometric evaluation of cell cycle distribution in CuB- or vehicle-treated H1299 cells was performed as described previously [36]. The population of cells in each cell-cycle phase was determined using Flow Jo software (Becton Dickinson).

2.8. Assays for apoptosis

Flow cytometric analysis: Apoptotic populations of vehicle- or CuB-treated cells were quantified using the dual staining annexin V-FITC/PI apoptosis detection kit (Invitrogen, Grand Island, NY) as per manufacturer’s instructions. Morphological examination: Vehicle- and CuB-treated cells were stained with 1:1 mixture of ethidium bromide (EB) and acridine orange (AO) (5 μg/ml each) for 5 min, and then analyzed for morphological changes under a fluorescence microscope (Nikon Eclipse TS100).

2.9. Western-blot analysis

Western-blot analysis was done as previously described [36]. Blots were probed for Bcl2, Bax, CyclinB1, cleaved caspase-3, Cleaved PARP, PCNA, pSTAT3, STAT3, pHS27, p73, p53, p21, pRho, pJNK, Rac, Cdc42, ERK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and gelsolin antibody (R&D systems, Minneapolis, MN). β-Actin (Sigma, St. Louis, MO) was used as loading control.

2.10. Determination of GSH and protein thiold content

GSH levels in H1299 cells treated with vehicle or 0.35 μM CuB in the presence and absence of 5 mM NAC for 1 and 4 h was determined with GSH/CSG-Glo™ assay kit (Promega, Madison, WI) as per manufacturer’s instructions. Cellular protein thiold content was measured as described [13]. Briefly, post-treatment, H1299 cell pellets (obtained after deproteinization with sulfosalicylic acid) were finally resuspended in 0.5 M Tris-HCl (pH 7.6). DTNB (100 μM) was then added and 30 min after the absorbance was measured at 410 nm. The protein concentrations of the solutions were determined using BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) and the thiold content of each sample was normalized to their respective protein content.

2.11. Determination of CuB-NAC binding in vitro

To evaluate CuB-NAC interaction, increasing concentrations of NAC (0.98, 1.9 and 3.9 mM) were added to 100 μM of CuB (9 μM) in a 96-well microplate. The intrinsic absorbance of CuB was monitored by an absorbance scan at 200–450 nm using M2 spectramax spectrophotometer. A red-shift in the spectrum indicates complex formation.

2.12. Conjugation of CuB with NAC and GSH

CuB (5 mM stock in DMSO diluted 1:10 in PBS) was incubated with NAC (500 mM stock in DMSO diluted 1:100 in PBS) or GSH (5 mM stock in DMSO diluted 1:10 in PBS) for 10 min and 30 min at room temp, respectively. Post-incubation, the samples were further diluted 1:10 in methanol and analyzed by direct injection using nano-spray (TriVersa NanoMate, Advion Biosciences, Ithaca, NY) on a hybrid linear ion trap FT-ICR-MS (Fourier-transform ion cyclotron resonance mass spectrometer, Finnigan LTQ FT, Thermo Electron, Bremen, Germany). The electron-spray ionization was performed in positive ion mode by applying 1.85 kV with no head pressure. MS runs were recorded over a mass range from 100 to 1000 Daltons. MS/MS analysis was done on select ions to confirm adduct formation.

2.13. Statistical analysis

Statistical differences between groups were assessed by Students t-test. Differences between mean were considered significant if p < 0.05.

3. Results

3.1. CuB suppresses the growth of lung cancer cells in vitro and in vivo

CuB dose-dependently inhibited the proliferation of all NSCLC cells (H1299, A549, H661 and HCC-827) irrespective of their p53 or EGFGR status; time-dependent inhibition was also evident as explored. As shown in Fig. 1c and 1d, CuB significantly blunted H1299 xenograft growth in nude mice was further explored. As shown in Fig. 1c and 1d, CuB significantly blunted H1299 xenograft growth in nude mice was further explored. As shown in Fig. 1c and 1d, CuB significantly blunted H1299 xenograft growth in nude mice was further explored. As shown in Fig. 1c and 1d, CuB significantly blunted H1299 xenograft growth in nude mice was further explored.
the appearance of moderate to severe surface–plasma membrane protrusions, called blebs, following 30 min of CuB treatment (Fig. 2a). A correlation between surface bleb formation and perturbation of normal actin cytoskeletal organization is reported [3] hence, the effect of CuB on F-actin cytoskeletal microfilaments in H1299 cells was analyzed. A 2 h CuB treatment dramatically changed the distribution of F-actin from the homogeneous network in control cells to large intensely-fluorescent cytoplasmic aggregates, pointing to an increase in F-actin polymerization/stabilization (Fig. 2b(i)). Evaluation of signaling molecules (Fig. 2c) demonstrated early activation of p38 MAPK which led to an increase in the phosphorylation of the F-actin polymerization modulator Hsp27 that could be in part responsible for the observed polymerization and stability of the F-actin microfilaments [29]. Further verification for involvement of p38 MAPK signaling was obtained with the use of p38 MAPK inhibitor SB203580, which mostly prevented CuB-induced F-actin polymerization (Fig. 2b(ii)).

Actin-microfilament remodeling has also been shown to be regulated either by the Rho-family of GTPases or the ROS [12,17], hence the effect of CuB on Rho-family signaling molecules (Rac, Rho and cdc42) and intracellular ROS levels was further evaluated. As shown in Fig. 2d, while CuB had no obvious effect on the levels of RhoA, Rac, and cdc42 in the early time points (15 min, 2 h), downregulation of both pRac and cdc2 was observed following 8 and 24 h of CuB treatment. Additionally, up to 3 h post-CuB treatment except at very high (10 μM) CuB concentration, no increase in intracellular ROS levels was observed at 0.1 μM concentration at which CuB elicited the cytoskeletal changes (Fig. 2e). These data suggest the possible unlikeliness of the Rho family GT-Pases and intracellular ROS levels in mediating CuB-induced actin-cytoskeletal changes at the earlier time points.

3.3. CuB induces G2/M cell cycle arrest and mitochondrial apoptosis

CuB has been shown to inhibit G2/M cell-cycle transition and induce mitochondrial apoptosis in other cell types [8,41]. Flow-cytometric analysis of CuB-treated H1299 cells showed an early and sustained G2/M cell arrest (40–60%) throughout the treatment
Fig. 2. Effect of CuB on cellular morphology actin cytoskeleton and intracellular ROS generation. (a) Morphologic changes in H1299 cells treated with CuB compared with vehicle treatment (magnification 20×). (b) (i and ii) Representative confocal images of F-actin assembly in H1299 cells exposed to (i) vehicle or CuB (ii) vehicle or CuB after 30 min pre-incubation with 10 μM of p38 MAPK inhibitor SB203580 at 100× magnification. (c) Immuno-blot analysis of MAPKs after 15 min and 2 h vehicle and CuB treatment, respectively. The phosphorylated proteins were normalized with their corresponding total proteins. β-actin has been shown for equal loading. Numbers above band depict changes in phosphorylated protein levels relative to the corresponding DMSO-treated controls. (d) Immuno-blot analysis of Rho-family GTPases as mediators of cytoskeletal alterations after vehicle and CuB treatment, respectively. (e) Early H₂O₂ accumulation in H1299 cells exposed to 0.1, 0.5 and 10 μM concentrations of CuB. The relative H₂O₂ production is expressed as the % increase in fluorescence in CuB-treated cells compared to cells exposed to PBS alone. Bars represent SD of 3 independent experiments performed in triplicates. a, p < 0.05, and b, p < 0.005 compared to vehicle treatment. *Fold change values shown for phosphorylated proteins only.
duration (Fig. 3a). Additionally, apoptotic populations of 10% and 14% at 24 h and 22% and 33% at 48 h were also observed following 0.1 μM and 0.35 μM of CuB-treatment, respectively (Fig. 3b). Validation for apoptosis was further obtained by AO/EB staining to detect apoptotic-morphological alterations such as membrane blebbing, chromatin aggregation and nuclear condensation (Fig. 3c). Similar effects of CuB on cell cycle arrest and apoptosis were also observed in A549 cells (data not shown).

Evaluation of signaling molecules revealed a dose- and time-dependent decrease in the activation of ERK and STAT3 proteins, induction in the levels of cell cycle regulatory cyclin kinase inhibitor, p21 (WAF1) and down regulation of cyclin B1, cdc2p34 (Cdk1), cyclin D1 and PCNA proteins (Fig. 3d(i and ii)). Furthermore, a decrease in the expression of anti-apoptotic protein Bcl2 accompanied by an increase in pro-apoptotic Bax, an increase in the cytosolic levels of cytochrome c, a significant and progressive activation of caspase-9 and caspase-3 and cleavage of their downstream targets PARP and gelsolin were also observed (Fig. 3e). These data collectively suggest that CuB alters the levels of cellular proteins to induce G2/M arrest and mitochondrial apoptosis in the cells.

Additionally, the expression levels of these proteins in the tumor lysates of the CuB-treated mice were also analyzed. In corroboration with the in vitro studies, a similar modulation in expression of the biomarkers was observed (Fig. 3f(i and ii) and g(i and ii)).

3.4. CuB's cytotoxicity and other cellular effects are blocked by the thiol antioxidant NAC and not vitamin C and ascorbic acid

The dose response for NAC chemoprotection was evaluated in H1299 cells treated concurrently with varying concentrations of NAC (0.1, 1, 5 and 10 mM) and CuB (0.1–1 μM) for 72 h. NAC significantly protected cells from CuB-induced cytotoxicity dose-dependently (Fig. 4a). Notably, other antioxidants ascorbic acid and “vitamin E” at 0.2 mM concentration failed to afford much protection (data not shown). To further comprehend the protective role of NAC, the effect of NAC adding time on CuB's cytotoxicity was assessed (Fig. 4b). Incubating cells with a mixture of 0.35 μM CuB and 5 mM NAC or a 2 h pretreatment with 5 mM NAC followed by addition of 0.35 μM CuB in the continued presence of NAC afforded complete protection. However, removal of NAC prior to the addition of CuB or addition of NAC, 2 h post-treatment with CuB offered either null or minimal protection, thus suggesting (i) the need for concurrent presence of both NAC and CuB to prevent CuB's cytotoxicity and (ii) irreversibility of CuB's effects.

Because CuB's cytotoxic effects were mediated by the onset of cellular morphological changes, actin-cytoskeletal alterations, G2/M arrest and apoptosis, the effect of NAC (5 mM) on these events was explored. As shown in Fig. 4c–e, NAC completely blocked all the cellular processes modulated by CuB.

3.5. CuB-induced depletion of intracellular thiol content and alters cellular redox balance

In the absence of significant ROS generation and preferential inhibition of CuB's cytotoxicity by the thiol antioxidant NAC, we sought to determine CuB's effects on the levels of intracellular thiols in H1299 cells. Following treatment with 0.35 μM CuB, an increase in GSSG (108% at 1 h and 131% at 4 h) paralleled the loss of GSH (92% at 1 h and 79% at 4 h) (Fig. 5a, data shown only for 4 h), causing a decrease in the GSH/GSSG ratio of 6.95 at 1 h and 4.51 at 4 h as compared to 8.74 in vehicle-treated sample (Fig. 5b). Additionally, a rapid and significant depletion of protein thiols was also observed following 1 h CuB treatment (Fig. 5c) and all the above responses were partially or completely prevented by NAC (Fig. 5a–c).

Further verification of the vital role of GSH depletion in CuB's cytotoxic effects was obtained when a significant enhancement in CuB's cytotoxicity was seen following a 4 h pre-treatment of H1299 cells with 5 mM concentration of the glutathione synthesis inhibitor BSO (Fig. 5c). These findings thus suggest a critical role for cellular thiols in the development of CuB's cytotoxicity. To rule out the possibility that the observed effects of CuB on the thiols and subsequent protection of its cytotoxicity by NAC was a cell linespecific effect, both A549 and HCC-827 cells were also treated with CuB in the presence and absence of NAC and/or BSO which essentially resulted in a similar trend in effects (Fig. 5d and e), thus suggesting the universality of CuB's effect.

3.6. NAC and GSH interact with CuB in a cell-free system

Since the concurrent presence of both NAC and CuB in the cell culture medium was necessary to prevent CuB's cytotoxic effects, the UV absorption characteristics of CuB in the presence and absence of NAC was analyzed to determine a possible interaction between the two. NAC exerted both a hypochromic effect on CuB absorption and a bathochromic shift in λmax of CuB. A dose-dependent decrease in the absorption intensity of CuB from 2.1 to 1.4, 1.1 and 0.65 with 0.98, 1.9 and 3.9 mM of NAC, respectively, and a shift in λmax of CuB from 212 nm to 220 nm in the presence of NAC were observed thus suggesting that NAC does interact and bind with CuB (Fig. 6a). Similar effects were observed with GSH; however vitamin E and ascorbic acid did not influence the absorption characteristics of CuB (data not shown).

To further confirm the CuB-thiol interaction, CuB was incubated with NAC and GSH respectively, and the masses of the individual compounds and the resulting adducts were examined using FTICR mass spectrometry (Fig. 6b and c). A major peak was obtained for CuB with the addition of 1Na+ (m/z 581.31), NAC with the addition of 1Na+ (m/z 186.02) and GSH with the addition of 1H+ (m/z 308.09) respectively. Furthermore, one major adduct with NAC at m/z 744.339 [CuB + NAC + Na]+ and 2 probable adducts with GSH at m/z 866.413 [CuB + GSH + Na]+ and m/z 888.396 [CuB + GSH + Na]+, were also detected. While the reaction between CuB with NAC was very fast and gave one abundant adduct peak, its reaction with GSH was slow and resulted in less enriched adduct peaks. It is worthwhile to mention here that the complex of CuB with NAC was however not detectable by HPLC-UV, presumably due to either the low sensitivity of HPLC or due to disintegration of the hydrophobic interaction between CuB and NAC under the chromatographic conditions.

4. Discussion

In the current study, we demonstrate that CuB is a potent suppressor of human NSCLC cell growth both in vitro and in vivo through its effects on actin-cytoskeleton, MAPK, STAT3, cell-cycle regulatory and apoptotic signaling. A direct role of p38 MAPK/Hsp27 signaling in mediating CuB-induced actin cytoskeletal alterations is further demonstrated. We also show for the first time that CuB disrupts cellular redox status by depleting cellular GSH and protein thiols levels and cellular GSH/GSSG ratios and that this decrease in GSH levels is probably due to direct reaction of CuB with GSH as evidenced by UV and FTICR mass spectrometric approaches.

A plethora of recent reports suggest pivotal role for cellular redox status in the regulation of cell growth and function [10,43]. In addition to ROS, intracellular GSH/GSSG ratio and protein thiols are key redox regulators that are crucial for multiple biological functions, including actin cytoskeletal remodeling [37], cell cycle progression [44], mitochondrial apoptosis [15] and regulation of
redox sensitive signal transduction proteins and transcription factors [9,40]. Furthermore, Franco et al. has also demonstrated that alterations in cellular thiol levels can trigger apoptosis independently of ROS [15]. In view of these reports and coupled with (i) the lack of ROS involvement in this study, (ii) significant depletion of cellular thiol levels, and (iii) exaggeration of CuB's
cytotoxicity by the glutathione synthesis inhibitor BSO, it is therefore reasonable to postulate that CuB-induced modulation of intracellular thiols might be amenable for its downstream effects. While the observed effects of CuB on actin cytoskeleton, cell cycle and apoptosis is in concordance with previously published reports [8,42,49], the inability of CuB to induce intracellular ROS levels, however, is in disagreement with the study by Yasuda et al. [48] which probably could be attributed to differences in cell types and the time points analyzed. While Yasuda et al. measured intracellular ROS levels following 24 h of CuB exposure, we measured early ROS levels following 0–3 h exposure to CuB, to preferentially account for its role in the initiation of CuB’s cellular effects.

An intriguing observation that remains central to our study and other studies by Yasuda et al. [48] and Zhang et al. [50] though, is the complete protection of CuB’s biological effects by the thiol antioxidant NAC. While the protective effects of NAC in those studies are attributed to its antioxidant effects either by decreasing CuB-induced cellular ROS levels [48] or basal cellular ROS levels [50], we postulate that NAC being a thiol nucleophile, (1) can interact with CuB in the extracellular medium to form a CuB–NAC adduct or (2) compete with cellular thiols targeted by CuB, thus leading to the subsequent loss of CuB’s biological effects.

A fundamental question, which remains unclear though, is how CuB interacts with the cellular thiols and modifies their levels? According to previous reports, certain drugs can bind intracellular GSH and protein thiols and thereby decrease their levels [2,5,33,39]. Additionally, some cyclopentenone prostaglandins and sesquiterpene lactones have also been shown to react with a...
thiol nucleophile group via Michael-type addition due to the presence of α,β-unsaturated ketone or carbonyl moieties in their structure [2,5]. Since CuB also possesses a similar α,β-unsaturated ketone moiety in its structure, the presence of such functional group might render it susceptible to binding with thiols. Although similar speculations have been made by Whitehouse and Doskotch [47] and Kupchan and Tsou [28], conclusive evidence for this notion comes from our UV and mass spectrometric studies where we demonstrate a direct interaction of CuB with thiol nucleophiles, such as GSH and NAC, through the identification of the respective adducts.

Many drugs and chemicals that form reactive electrophiles also modify protein structure by binding to the functional –SH groups.
Fig. 6. CuB interacts with NAC and GSH. (a) Absorption spectra of CuB in the presence and absence of NAC in a cell-free system. Arrows point to absorbance of CuB and the dotted lines show the $\lambda_{	ext{max}}$ of CuB in the absence and presence of NAC respectively. (b and c) CuB was incubated with NAC for 10 min and GSH for 30 min at room temp respectively and the mixture was resolved by FTICR mass spectrometry. Masses were recorded in positive ion mode (to save space and for clarity of the results, patterns presented have been truncated). (d) Structure of Cucurbitacin B (shaded region shows the site for nucleophilic attack as proposed by Kupchan and Tsou [28]. Adapted partially with permission from Kupchan S.M. and Tsou G. Tumor Inhibitors. LXXXI. Structure and partial synthesis of fabacein. J. Org. Chem. 1973; 38: 1055–1056. Copyright (1973), American Chemical Society.
in their cysteine moieties [6]. Additionally, changes in cellular redox status can also lead to the modification of protein structure [19,32]. Considering that fact that the vast array of protein targets of CuB such as JAK2, STAT3, NF-kB, p53, cyclinD1, p21 and cdc2 are susceptible to electrophile-induced structural modifications [5,23,25,31,35,46], we speculate that CuB’s effects on its various cellular protein targets could also be facilitated either (i) primarily by direct interaction of CuB with the functional nucleophilic groups of these proteins or (ii) secondarily through its effects on cellular GSH which subsequently might lead to the structural and functional modification of the client proteins. Even though further studies are warranted to explore these possibilities and identify the putative protein targets for covalent modification by CuB, nevertheless data presented in this paper demonstrate for the first time that CuB-induced cytotoxicity are due to alterations in cellular redox balance which, in turn, might be a consequence of CuB’s reactivity with the cellular nucleophiles such as GSH and the protein thiols.

Our study thus demonstrates the therapeutic potential of CuB in NSCLC and advances our understanding into the working mechanisms of this potential future drug.

Acknowledgements

This work was supported from Agnes Brown Duggan Endowment, James Graham Brown Cancer Center funds and Kentucky Lung Cancer Research Program cycles 7 and 10. R.C.G. holds the Agnes Brown Duggan Chair in Oncological Research. Drs. Tarig Hamid, Srivani Ravoori and Madhavi Rane are acknowledged for useful discussions during the course of the work. Finally, we also thank Dr. Michelle T. Barati and Dr. Rick Higashi for their help with the confocal and FITC/CR mass-spectrometry studies, respectively.

References


