Clioquinol induces cytoplasmic clearance of the X-linked inhibitor of apoptosis protein (XIAP): therapeutic indication for prostate cancer

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Clioquinol (5-chloro-7-iodo-8-quinolinol) is a copper ionophore that was used primarily during the 1950–1970s as an oral anti-microbial agent. It has been established that clioquinol displays toxicity towards malignant cells, inducing caspase-dependent apoptosis. In the present study we therefore investigated the effect of clioquinol on the XIAP [X-linked IAP (inhibitor of apoptosis protein)], as one of its primary functions is to hinder caspase activity and suppress apoptotic cell death. Clioquinol treatment caused cytoplasmic XIAP to rapidly relocate to the nucleus in multiple human transformed (hyperplastic and carcinoma) prostate lines. Clioquinol also caused the cytoplasmic clearance of other IAP family members (cIAP1 and cIAP2). Copper, and no other relevant bivalent metal (e.g. zinc or iron), was exclusively required for clioquinol to elicit an effect on XIAP. We further demonstrated that clioquinol selectively targets and rapidly destroys transformed prostate lines without harming primary prostate epithelial cells. The toxicity of clioquinol was copper-dependent, positively correlated with the level of extracellular copper and could be abrogated by using the copper chelator TTM (tetrathiomolybdate). Clioquinol forced the profound accumulation of intracellular copper with ensuing toxicity influenced by key regulators of cellular copper homeostasis. Taken together, our results provide significant insight into clioquinol toxicity and reveal an exciting therapeutic approach for the treatment of prostate cancer.

Key words: cancer, clioquinol, copper, prostate, X-linked inhibitor of apoptosis protein (XIAP).

INTRODUCTION

Metal dyshomeostasis in cancer patients has been well documented and forms the basis of current investigations into therapeutic intervention [1–5]. Elevated copper in both malignant tissue and serum is emerging as a hallmark of cancer, having been established in a range of cancer types including breast, ovarian, cervical, lung, stomach and leukaemia (reviewed in [5]). Conversely, zinc and iron concentrations are often significantly reduced [5–7]. Copper is essential for the development and progression of cancer [1,2,8,9]. The copper chelator TTM (tetrathiomolybdate) and its analogues suppress tumour growth, angiogenesis and metastases and are being investigated in clinical trials [2–4]. Chelation of copper alone, however, is insufficient to kill malignant cells, necessitating its use in an adjunctive manner to be an effective therapeutic option [1–4]. Clioquinol (5-chloro-7-iodo-8-quinolinol), an 8-hydroxyquinoline derivative with copper ionophoric properties, was shown previously to be toxic towards malignant cell lines both in vitro [10–13] and in vivo [12]. Distinct from the sequestering nature of a chelator, an ionophore by definition transports specific metal(s) into cells often allowing them to become bioavailable. Clioquinol transports both copper and zinc into mammalian cells [14,15] and causes the death of malignant cells by inhibiting the proteasomal system and by inducing apoptosis through a caspase-dependent pathway [10,12,13,16,17]. While the basis for the selectivity towards malignant cells is not known, clioquinol toxicity is copper-dependent and exacerbated by increasing the cation level [16]. Elevated copper in malignant cells may predispose them to clioquinol toxicity, but this has not been confirmed. Additionally, clioquinol can potentiate the anti-tumour effects of other drugs such as docosahexaenoic acid [11].

Clioquinol-induced death of malignant cells is initiated by inhibition of the proteasomal system, specifically inhibition of chymotrypsin-like activity [10,12,13,17]. In malignant cells, inhibition of chymotrypsin-like activity represents a pro-apoptotic stimulus, causing caspase activation and subsequent cell death [12,17–19]. Accordingly, the toxicity of clioquinol towards malignant cells can be abrogated by the caspase pan-inhibitor z-VAD-fmk (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) [12,17]. Important modulators of caspase activity are the IAPs (inhibitor of apoptosis proteins), which selectively bind and inhibit caspases to block cell death [20]. Of the eight members of the IAP family, XIAP (X-linked IAP) has received the most attention. XIAP is expressed in most tissues and is also the most potent inhibitor of caspases (3, 7 and 9), having Ki values of 0.2–0.8 nM compared with 1–20 nM for other IAP family members [21,22]. The elevated expression of XIAP in cancerous cells has been shown to correlate with chemoresistance and poor clinical outcome in patients [23–25]. Furthermore, XIAP knockdown in vitro has been demonstrated to result in caspase activation and apoptosis in several malignant cell lines [26,27].

IAPs are metalloproteins containing one to three BIR (baculoviral IAP repeat) motifs that co-ordinate zinc and form functionally distinct structures [20]. The BIR domains are invariably located within the N-terminus and mediate the inhibitory interactions with caspases. Several family members including XIAP also contain a C-terminal zinc-based RING domain, which has E3 ligase activity [20]. XIAP has been shown to directly bind copper and may play a role in cellular copper homeostasis/transport

Abbreviations used: ATP7B, Cu-transporting ATPase 7B; BIR, baculoviral inhibitor of apoptosis protein repeat; cIAP, cellular inhibitor of apoptosis protein; FBS, fetal bovine serum; HA, haemagglutinin; HRP, horseradish peroxidase; IAP, inhibitor of apoptosis protein; NLS, nuclear localization signal; PARP, poly (ADP-ribose) polymerase; PBS-T, PBS with 0.1% Tween 20; PEG, prostate epithelial cell; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SOD, superoxide dismutase-1; TPEN, N,N,N′′′-tetraakis-(2-pyridyldimethyl)ethylenediamine; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; TTM, tetrathiomolybdate; wt, wild-type; XIAP, X-linked IAP.

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producers of copper, caused the partial degradation of XIAP and its rapid relocation from the cytoplasm to the nucleus. Clioquinol also depleted the cytoplasm of other IAP family members (cIAP1 and cIAP2), thereby providing a molecular explanation of how clioquinol induces caspase activation in malignant cells. Furthermore, we have demonstrated that clioquinol is highly toxic to a diverse panel of transformed prostate cell lines. By contrast, the viability of primary PrECs (prostate epithelial cells) was unaffected by clioquinol treatment, as was the cytoplasmic level of XIAP in these cells. The toxicity of clioquinol was dependent on the level of milieul copper and was influenced by the key cellular regulators of copper homeostasis, ATP7B and Ctrl1. Our results provide significant insight into the toxicity of clioquinol, and the therapeutic implications for prostate cancer are discussed.

EXPERIMENTAL

Cell lines and reagents

Prostate hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LnCaP) cell lines were cultured at 37°C (5% CO2) in RPMI 1640 medium (Invitrogen), supplemented with 10% (v/v) FBS (fetal bovine serum; SAFC Biosciences). Primary human PrECs were cultured at 37°C (5% CO2) in serum-free PrEGM™ medium, containing the supplements and growth factors provided by the manufacturer (Millennium Bioscience). Both RPMI 1640- and PrEGM™-based media contain less than 1 μM of copper (results not shown). PrECs were supplied cryopreserved in second passage, allowing establishment of proliferative cultures that can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passaged several times. Clioquinol, TTM and resazurin can be passed through the BAP3 transporter ATP7B (Cu¬transporting ATPase 7B) [30]. The copper chaperone CCS [Cu2+ chaperone of SOD1 (superoxide dismutase-1)] also undergoes XIAP-mediated ubiquitination, which enhances its ability to deliver copper to its cytoplasmic target SOD1 [28]. Furthermore, tissues derived from XIAP-deficient mice were found to be copper-deficient [30]. XIAP may also mediate apoptotic cell death in copper toxicity disorders, with pathological levels of copper causing its degradation and dissociation from caspases [29].

We report in the present paper that treatment of transformed (hyperplasic and carcinoma) prostate cells with the copper ionophore clioquinol, in the context of physiological levels of copper, caused the partial degradation of XIAP and its rapid relocation from the cytoplasm to the nucleus. Clioquinol also depleted the cytoplasm of other IAP family members (cIAP1 and cIAP2), thereby providing a molecular explanation of how clioquinol induces caspase activation in malignant cells. Furthermore, we have demonstrated that clioquinol is highly toxic to a diverse panel of transformed prostate cell lines. By contrast, the viability of primary PrECs (prostate epithelial cells) was unaffected by clioquinol treatment, as was the cytoplasmic level of XIAP in these cells. The toxicity of clioquinol was dependent on the level of milieul copper and was influenced by the key cellular regulators of copper homeostasis, ATP7B and Ctrl1. Our results provide significant insight into the toxicity of clioquinol, and the therapeutic implications for prostate cancer are discussed.

Plasmid constructs and transfection

The HA-tagged XIAP constructs (XIAP-Full, BIR1-2-3, BIR1-2, BIR2-3, and ΔBIR) were kindly provided by Associate Professor Colin Duckett (Division of Molecular Medicine and Genetics, University of Michigan Medical School, Ann Arbor, M.I., U.S.A.) [31]. The generation of Myc-tagged Ctrl1 has been described previously [32]. FLAG-tagged ATP7B was a gift from Dr Sharon La Fontaine (School of Life and Environmental Sciences, Deakin University, Melbourne, Australia). FLAG-tagged wtXAPC7 (wt is wild-type) was generated through PCR amplification of cDNA using the forward (5'-CTCTTCAAGCTTGATCCAGGCTACGACCGCGCCATCACC-3') and reverse (5'-CTCTTCCGAAATTCCTAATCTATCATCATATCTATATGCTAGCTCTTCTTTGTTCCTTCC-3') oligonucleotides and by incorporation into the mammalian expression vector pcDNA3. The forward oligonucleotide introduced a BamHI endonuclease restriction site 5' to the start codon. The reverse oligonucleotide introduced sequence encoding theFLAG epitope in-frame immediately before the stop codon and a flanking 3' EcoRI endonuclease restriction site. Template cDNA was isolated from human SW480 colon adenocarcinoma cells using the TRIZol® Plus RNA Purification (Invitrogen) and the MMLV (Moloney murine leukaemia virus) reverse transcriptase (Promega) systems following the manufacturer's protocols. The PCR contained 1 × PCR buffer, 0.2 mM of each dNTP, 2 mM MgCl2, 0.2 μM of each primer, 2.5 units of Platinum Taq DNA polymerase and 3 μl of cDNA (Invitrogen). Reactions were run on a MultiGene Gradient Thermal Cycler (Labnet) on the following programme: one cycle of 94°C for 2 min, 38 cycles of 94°C for 45 s, 57°C for 60 s and 72°C for 60 s, followed by one cycle of 72°C for 2 min. The resultant PCR product was digested with BamHI and EcoRI and cloned into pcDNA3 at the same sites. The FLAG-tagged XAPC7del construct, that lacks the NLS (nuclear localization signal) sequence (amino acids 241–248), was generated using the above strategy, but with a substituted reverse primer (5'-CTCTTCCGAAATTCCTAATCTATCATCATATCTATATGCTAGCTCTTCTTTGTTCCTTCC-3'). This reverse primer introduced the C-terminal truncation (encoding amino acids 241–248) and a flanking 3' EcoRI endonuclease restriction site. The integrity of both wtXAPC7 and XAPC7del was confirmed by sequencing.

For transient transfections, prostate carcinoma PC3 cells were seeded into either 12- or 24-well plates and cultured for 16-24 h to allow adequate adhesion. Transfection of plasmid was performed with FuGENE® HD (Roche) following the manufacturer's protocol.

Lysate preparation and Western blot analysis

Cells were lysed using ice-cold lysis buffer [50 mM Tris/HCl (pH 6.8), 150 mM NaCl, 0.5% Nonidet P40 and protease inhibitor cocktail]. Total cell lysates were made up in SDS protein sample buffer (final concentrations: 50 mM Tris/HCl, 2% (w/v) SDS, 0.1% Brommophenol Blue, 10% glycerol and 10% 2-mercaptoethanol). Alternatively, lysates were separated into cytoplasmic- and nuclear-enriched fractions as follows: total lysates were centrifuged at 10,000g for 10 min at 4°C and the supernatant/cytoplasmic fractions were transferred into fresh tubes. The nuclear-enriched pellets were washed twice by resuspension in lysis buffer and centrifugation (10,000g...
for 10 min at 4 °C). Each washed nuclear-enriched pellet was resuspended in 50 μl of SDS protein sample buffer. Protein samples were fractionated using the Mini-PROTEAN® Tetra System (Bio-Rad Laboratories) and transferred on to nitrocellulose membranes (Amersham Biosciences) using the XCellIII™ blot module and the Xcell SureLock™ mini-cell system (Invitrogen) according to the manufacturer’s instructions. The nitrocellulose membrane was then blocked using 5% (w/v) skimmed milk powder in PBS (1 h at room temperature, 22 °C) before being incubated with primary antibody diluted in PBS-T (PBS with 0.1% Tween 20) (1–2 h at room temperature). The following primary antibody dilutions were used: mouse anti-XIAP (diluted 1/1500); mouse anti-β-actin (diluted 1/1000); mouse monoclonal anti-HA (diluted 1/1000); mouse anti-histone H2B (diluted 1/100); rabbit anti-β-tubulin III (diluted 1/1000), rat anti-cIAP1 (diluted 1/1500); rat anti-cIAP2 (diluted 1/1000); rabbit anti-FLAG (diluted 1/5000); mouse anti-c-Myc (diluted 1/1000); mouse anti-caspase 3 (diluted 1/1000); rabbit anti-PARP (diluted 1/1000) and rabbit anti-PTEN (diluted 1/1000). Subsequently, the membranes were washed four times with PBS-T (5 min), before being incubated with the appropriate secondary antibody (1 h at room temperature). The following secondary antibody dilutions were used: IRDye 680LT-conjugated goat anti-mouse IgG (diluted 1/5000); IRDye 800CW goat anti-rabbit IgG (diluted 1/5000); HRP-conjugated rabbit anti-rat IgG (diluted 1/2000) and HRP-conjugated goat anti-rabbit IgG (diluted 1/5000). Analysis was performed with the Odyssey® Infrared Imaging System (LI-COR), or by using the Western Lightning™ Plus-ECL (enhanced chemiluminescence; PerkinElmer), according to the manufacturer’s instructions. Densitometric analysis was used to evaluate immunolabelled protein intensity. The pixel intensities (arbitrary units) were quantified using Multi Gauge software (Fujifilm) and levels were normalized against a loading control of either β-actin or histone H2B. At least three independent experiments were used for all comparisons.

**Metabolic activity assay**

Prostate carcinoma PC3 cells were cultured for 16–24 h to allow adequate cellular adhesion. The medium was then replaced at 2-h intervals with medium supplemented with clioquinol alone, CuCl2 alone, or with the combination of both clioquinol and CuCl2, the concentrations of which are shown in the Results section. Clioquinol was freshly made at each treatment interval. To each well, 200 μl (10% v/v) of resazurin (alamarBlue®) was added and incubated with the cells for the last hour of treatment. The reduction of resazurin was measured using a POLARStar OPTIMA multidetection microplate reader (BMG Labtech) with excitation and emission wavelengths of 540 nm and 610 nm respectively. The mean of quadruplicate determinations for each test condition with standard deviations (± S.D.) were used for comparisons.

**Cell viability assay**

Cell viability was measured by propidium iodide (Merck) dye-exclusion viability assay and FACs. The prostate hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cells were seeded separately into 12-well trays and were cultured for 16–24 h to allow adequate cellular adhesion. The cells were treated as outlined in the Results section. Following treatment, the conditioned medium was collected and transferred into 5 ml FACs tubes. Adhered cells were harvested using 1 ml of trypsin solution (0.025% trypsin and 0.02% EDTA) for 5 min (incubated at 37°C) and then combined with their corresponding conditioned medium. Cells were pelleted by centrifugation at 1500 g for 5 min and 4°C, supernatant was aspirated and the cells were resuspended in 1 ml of PBS containing 5 μg/ml (7.4 μM) propidium iodide (or neat PBS for control). The proportion of dead cells was measured on a FACS Analyzer (BD FACSCANTO™II) with 10000 events measured. The means of quadruplicate determinations for each test condition with standard deviations (± S.D.) were used for comparisons.

**Intracellular metal analysis**

The metal content of prostate hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cells was measured under basal conditions, or subsequent to treatment as outlined in the Results section. To harvest cells, the medium was discarded and the cells were washed with 2 ml of trypsin solution (0.025% trypsin and 0.02% EDTA) before being harvested with 1.6 ml of trypsin solution. The cells were counted using a Beckman Coulter Z Series Cell Count and Size Analyzer. A 1.5 ml aliquot of the cell suspension was centrifuged at 1000 g for 5 min at room temperature to pellet the cells, after which the supernatant was removed. The cellular metal concentration was measured using inductive-coupled plasma MS (Agilent 7700, Varian). The mean of quadruplicate determinations for each test condition with standard deviations (± S.D.) were used for comparison.

**Immunofluorescence microscopy**

PC3 cells were seeded on to flame-sterilized 13 mm glass coverslips in a 24-well tray and were cultured until ~80% confluent. Cells were treated for 6 h with either fresh medium (containing 0.2% DMSO) or medium supplemented with clioquinol (10 μM) alone, CuCl2 (20 μM) alone, or a combination of both clioquinol (10 μM) and CuCl2 (20 μM). Following treatment, the cells were fixed in 4% (w/v) paraformaldehyde for 10 min at room temperature, permeabilized using 0.1% Triton X-100 for 10 min at room temperature and then blocked with 1% (w/v) BSA and 1% (w/v) gelatin at 4°C overnight. The coverslips were then incubated with mouse monoclonal anti-XIAP (1/50 dilution) for 1 h at room temperature, then incubated with goat anti-mouse IgG conjugated to Alexa Fluor® 488 (1:2000 dilution) for 1 h at room temperature. Coverslips were mounted on to glass slides using FluorSave™ Reagent (Calbiochem) and cells were analysed using an Olympus BX-51 microscope and a 60× water-objective lens.

**RESULTS**

**Clioquinol selectively targets transformed prostate cells**

The copper ionophore clioquinol has been shown to be toxic for several malignant cell types including prostate cancer cells [10–13,16,17]. However, a comparative study between normal and malignant prostate cells has not been carried out. Therefore we sought to determine whether clioquinol was a feasible therapeutic option for prostate cancer by comparing the relative sensitivity of normal (PrECs) with transformed (hyperplasic and carcinoma) prostate cells to clioquinol treatment. Initially we investigated the effect of clioquinol and copper on the metabolic activity of prostate carcinoma PC3 cells (Figure 1A). Metabolic activity was determined by measuring the intracellular conversion (reduction) of the cell-permeable dye resazurin (alamarBlue®) to the fluorescent resorufin molecule (Figure 1A). The treatment of PC3 cells with either a physiological level of copper (10–20 μM) [33] or an elevated pathological level of copper (40 μM) [33],
Clioquinol with copper induces the nuclear accumulation of XIAP

XIAP is a potent anti-apoptotic protein that inhibits caspases and suppresses apoptosis at the final juncture [21,22]. To better understand how clioquinol induces apoptotic cell death of malignant cells, we investigated the effect of clioquinol on XIAP expression and its subcellular localization (Figure 2). The combined treatment of clioquinol and copper reduced the cytoplasmic level of XIAP, whereas treatment with either clioquinol or copper alone had no effect (Figure 2A). There was no marked difference in the rate of XIAP reduction when clioquinol was co-incubated with either a physiological level of copper (20 μM), or with an elevated pathological level of copper (40 μM) as found in the serum of patients with prostate cancer [5,34]. Copper can bind to proteins, changing their structure and by doing so often occlude antibody recognition. Since copper binds directly to XIAP [28], it was important to verify that the XIAP reduction observed (Figure 2A) was not due to antibody obstruction caused by copper and/or clioquinol co-ordination. For this purpose, we exogenously expressed an N-terminal HA-tagged XIAP protein in PC3 cells, and demonstrated that its cytoplasmic level was likewise reduced following combined clioquinol and copper treatment (Figure 2B). Immunofluorescence microscopy revealed that clioquinol and copper actually modifies the cellular distribution of endogenous XIAP from a dispersed cytoplasmic localization to a predominant nuclear localization (Figure 2C). Treatment with clioquinol or copper alone had no effect on the subcellular localization of XIAP (Supplementary Figure 1 at http://www.BiochemJ.org/bj/436/bj4360481add.htm). Translocation to the nucleus would account for the cytoplasmic reduction of XIAP seen by Western blotting (Figures 2A and 2C).

did not affect their metabolic activity (Figure 1A). Likewise, a clioquinol concentration of 10 μM, which can be easily achieved in serum through oral administration, was also found to be insufficient to elicit an effect on cellular metabolism. However, when the same concentration of clioquinol was combined with incremental amounts of copper (10–40 μM) there was a steady and dose-dependent decrease in metabolic activity. This decrease was mirrored by an increase in cell death, as determined by the propidium iodide dye-exclusion viability assay (Figure 1B). We further verified that clioquinol and copper treatment effectively kills a wide spectrum of transformed prostate cell lines (Figure 1C). The hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cell lines were selected to represent the status/response and metastatic potential. Despite disparity in the treatment coincided with decreased levels of native caspase 3, PARP and β-actin were immunolabelled as described in the Experimental section.

Figure 1 Ionophoric copper specifically targets transformed prostate cells

(A) PC3 cells were treated for the indicated times with medium supplemented with clioquinol alone, CuCl2 alone, or with a combination of both clioquinol and CuCl2 (concentrations shown). Metabolic activity was determined by the resazurin (alamarBlue®) assay. (B) PC3 cells were treated for 16 h with either fresh medium (control) or medium supplemented with clioquinol (10 μM) alone, CuCl2 (40 μM) alone, or with clioquinol (10 μM) in combination with incremental amounts of CuCl2 (10–40 μM). Cell viability was determined by the propidium iodide dye-exclusion viability assay and shown as a percentage in comparison with the control. Values are means ± S.D. of quadruplicate determinations for each test condition. (C) The prostate primary (PrEC), hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cell lines were treated with medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM) for the indicated times. The treatment medium was replenished every 24 h where PrECs were treated for 1 week (168 h). Cell viability was determined by the propidium iodide dye-exclusion viability assay. (D) PC3 cells were treated for 16 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Total cell lysates were analysed by Western blotting and endogenous native caspase 3, PARP and β-actin were immunolabelled as described in the Experimental section.
Clioquinol depletes cytoplasmic XIAP

Figure 2  Clioquinol with copper induces the nuclear accumulation and partial degradation of XIAP

(A) PC3 cells were treated for the indicated time with either fresh medium (containing 0.2 % DMSO) or medium supplemented with clioquinol (10 μM) alone, CuCl2 (20 μM) alone, or a combination of both clioquinol (10 μM) and CuCl2 (20 μM). Cytoplasmic preparations were analysed by Western blotting, and endogenous XIAP and β-actin were immunolabelled as described in the Experimental section. (B) PC3 cells exogenously expressing an N-terminal HA-tagged XIAP construct were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Cytoplasmic preparations were analysed by Western blotting, and HA-tagged XIAP and β-actin were immunolabelled as described in the Experimental section. (C) Immunofluorescent microscopy of endogenous XIAP in PC3 cells treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Detection of XIAP was performed using a mouse anti-XIAP antibody (dilution 1/50) followed by a goat anti-mouse IgG antibody conjugated to Alexa Fluor® 488 (diluted 1/2000). Photographs were taken using a 60 x water objective with an Olympus BX51 microscope. (D) PC3 cells were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Cytoplasmic- and nuclear-enriched fractions were analysed by Western blotting, and endogenous XIAP, histone H2B and β-tubulin III were immunolabelled as described in the Experimental section. (E) PC3 cells were treated with medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM) for the indicated duration. Cytoplasmic- (i) and nuclear-enriched fractions (ii) were analysed by Western blotting. Endogenous XIAP, β-actin and histone H2B were all immunolabelled as described in the Experimental section. (F) PC3 cells were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Total cell lysates were analysed by Western blotting and XIAP and β-actin were immunolabelled as described in the Experimental section. (G) PC3 cells were treated for 6 h with medium supplemented with CuCl2 (20 μM) and incremental amounts of clioquinol (5–20 μM). Cytoplasmic preparations were analysed by Western blotting, and XIAP and β-actin were immunolabelled as described in the Experimental section. Values are means ± S.D. of quadruplicate determinations for each test condition.

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and 2B). We further demonstrated by Western blotting, that corresponding to the cytoplasmic reduction of XIAP was the appearance of XIAP in the nuclear-enriched fraction (Figure 2D). Further characterization revealed an almost linear relationship between XIAP reduction in the cytoplasm (Figure 2Ei) and XIAP accumulation in the nuclear-enriched fraction (Figure 2Eii). Increasing the concentration of clioquinol did not accelerate the clearance of XIAP from the cytoplasm (Figure 2F), consistent with a rate-limited process such as entry into the nucleus. To determine whether the majority of XIAP molecules accumulate in the nucleus upon treatment, we further examined whole-cell lysates representing the total cellular pool of XIAP (Figure 2G). In addition to causing the nuclear accumulation of XIAP, treatment with clioquinol and copper reduced the overall cellular level of XIAP by approximately 35%. Taken together, these results demonstrate that the ionophore clioquinol, when incubated with physiologically relevant levels of copper, causes the partial degradation of XIAP and its rapid relocation to the nucleus.

Clioquinol induces cytoplasmic clearance of the IAP family by increasing intracellular copper

We next examined whether clioquinol and copper treatment similarly affects XIAP in other prostate cell lines (Figure 3A). Analogous to our observations in PC3 cells, XIAP in the prostate hyperplastic (BPH-1) and other carcinoma (Du145 and LNCaP) lines responded to clioquinol and copper treatment, albeit to different degrees, by relocating out of the cytoplasm (Figures 3Ai–3Aiii). An interesting observation was that the more resilient cell line, Du145 (Figure 3Ai), seemingly had a lower rate of cytoplasmic XIAP clearance in comparison with the other lines (Figures 3Eii and 3Eiii). Consistent with PrECs being resistant to clioquinol and copper treatment (Figure 1C), the cytoplasmic level of XIAP was also unreceptive (Figure 3Ei), even when challenged with double the concentration of both clioquinol and copper and with the treatment time extended (24 h). We next investigated the impact of clioquinol and copper treatment on the expression and subcellular localization of other IAP family members (Figures 3B and 3C). Analogous to the effects on XIAP, treating PC3 cells with clioquinol and copper reduced the cytoplasmic level of cIAP1, but at a slightly higher rate (Figure 3B). The cytoplasmic level of cIAP2 was also reduced, but at a much lower rate than observed for both XIAP and cIAP1. Nevertheless, despite the apparent differences in cytoplasmic clearance rates, all three IAP family members (XIAP, cIAP1 and cIAP2) were cleared from the cytoplasm and accumulated in the nuclear-enriched fraction (Figure 3C). Clioquinol has been shown to strongly co-ordinate and mediate the cellular uptake of both zinc and copper [14,15]. Therefore we investigated whether or not other bivalent metals together with clioquinol can cause the nuclear accumulation and/or the degradation of XIAP (Figure 3D). We found that only in the presence of copper could clioquinol exert any effect on the level of XIAP. The concentration of each metal used in this experiment represents the upper limit of their normal physiological range found in the serum of healthy individuals [35]. Classical copper chelators, such as TTM, have recently received considerable attention in the cancer field for their ability to inhibit metastasis and tumour growth [1–4]. We investigated whether TTM has any effect on the cytoplasmic level of XIAP (Figure 3E). Unlike the ionophore clioquinol, the chelator TTM, when combined with physiological copper, had no effect on the cytoplasmic level of XIAP. To verify that clioquinol and TTM modulate cellular metal levels, as we would predict, the above experiment was repeated but this time ensuring intracellular metals were measured (Figure 3F). Clioquinol treatment bypasses the normal cellular regulatory mechanism(s) of copper uptake and increases the intracellular copper level approximately 9-fold higher than in the presence of copper alone. Conversely, TTM treatment restricted cellular copper uptake to approximately half that of cells treated solely with copper. Both clioquinol and TTM had no effect on the intracellular level of zinc and iron (Figure 3F), or on magnesium, manganese, calcium or selenium (Supplementary Figure 2 at http://www.BiochemJ.org/bj/436/bj4360481add.htm). Taken together, these results demonstrate that copper can be exclusively harnessed by the ionophore clioquinol to deplete the cytoplasmic level of the IAP family members. This reduction coincided with the profound accumulation of intracellular copper.

Cellular copper homoeostatic mechanisms influence sensitivity to treatment with clioquinol and copper

The basal intracellular level of copper, zinc and iron were measured in the prostate cell lines (Figure 4A). The transformed cell lines all harboured between 2- and 6-fold greater intracellular copper than primary PrECs. However, there was no correlation between intracellular copper level (Figure 4A) and the sensitivity of the different cell lines to clioquinol and copper treatment (Figure 3C). There was also no consistent difference in intracellular zinc or iron between the transformed lines and primary PrECs (Figure 4A). To investigate whether the copper transporters Ctrl1 and ATP7B influence sensitivity to clioquinol and copper, we exogenously overexpressed each protein in PC3 cells (Figures 4B and 4C). It has been demonstrated previously that Ctrl1 overexpression increases the cellular uptake of copper, whereas overexpression of ATP7B enhances copper efflux (reviewed in [35]). As shown by Western blotting, overexpression of either Ctrl1 or ATP7B does not directly affect the level of cytoplasmic XIAP (Figure 4B). However, increasing their levels had significant and opposing effects on clioquinol and copper sensitivity, with Ctrl1 resulting in increased sensitivity, whereas ATP7B conferred greater tolerance (Figure 4C). These results indicate that cellular copper homoeostatic mechanisms influence the cellular sensitivity to clioquinol and copper treatment.

Nuclear relocation of XIAP involves homoeostatic mechanisms

The effect of clioquinol and copper on various HA-tagged domains of XIAP was examined in PC3 cells (Figure 5A). Each exogenously expressed XIAP domain was reduced in the cytoplasm (Figure 5B) and accumulated in the nuclear-enriched fraction (Figure 5C) following treatment. To explain the ability of clioquinol and copper to target multiple regions of XIAP, we investigated the involvement of the ubiquitin–proteasome system. We hypothesized that the nuclear accumulation of XIAP may be coupled with the translocation of proteasomes to the nucleus. This hypothesis was based on several pieces of information: (i) the nuclear accumulation of proteasomes had been previously demonstrated to occur as a consequence of their direct inhibition [36,37]; and (ii) clioquinol is known to be a potent proteasomal inhibitor [10,13,17]. To investigate this idea, we surmised that the direct inhibition of proteasomes should also cause the accumulation of XIAP in the nucleus.
Clioquinol depletes cytoplasmic XIAP

Figure 3  Clioquinol induces cytoplasmic clearance of the IAP family by increasing intracellular copper

(A) Du145 (i), BPH-1 (ii) and LNCaP (iii) cell lines were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Primary PrECs (iv) were treated with fresh medium (containing 0.2 % DMSO), or with medium supplemented with clioquinol (10 μM) and CuCl2 (20 μM), or with double the concentration of both clioquinol (20 μM) and CuCl2 (40 μM), for 6 and 24 h. As a control, PC3 cells were also treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Cytoplasmic preparations were analysed by Western blotting. XIAP, β-actin, histone H2B and β-tubulin III were immunolabelled as described in the Experimental section. (B) PC3 cells were treated with medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM) for the indicated duration. Cytoplasmic preparations were analysed by Western blotting, and XIAP, cIAP1, cIAP2 and β-actin were immunolabelled as described in the Experimental section. (C) PC3 cells were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM). Cytoplasmic and nuclear-enriched fractions were analysed by Western blotting, and XIAP, cIAP1, cIAP2, histone H2B and β-tubulin III were immunolabelled as described in the Experimental section. (D) PC3 cells were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with clioquinol (10 μM) alone, or clioquinol (10 μM) in combination with either ZnCl2 (7.5 μM), CuCl2 (25 μM), FeCl2 (30 μM) or MgCl2 (500 μM). Cytoplasmic preparations were analysed by Western blotting. XIAP and β-actin were immunolabelled as described in the Experimental section. (E) PC3 cells were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl2 (20 μM), or with both TTM (20 μM) and CuCl2 (20 μM). Cytoplasmic preparations were analysed by Western blotting. XIAP and β-actin were immunolabelled as described in the Experimental section. (F) PC3 cells were treated for 6 h with medium supplemented with CuCl2 (20 μM) alone (containing 0.2 % DMSO) (Control) or with both clioquinol (10 μM) and CuCl2 (20 μM), or with both TTM (20 μM) and CuCl2 (20 μM). Intracellular metal content was measured using inductively-coupled-plasma-MS. Results are the means ± S.D. (n = 4) and are shown as ng of metal per 10^6 cells.

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which has a much shorter half-life, demonstrated that MG132 treatment did indeed inhibit proteasomal activity (Figure 5E). Taken together, these results suggest that MG132 treatment, and Thus by inference proteasomal inhibition, causes the cytosolic movement of XIAP and its subsequent accumulation in the nuclear-enriched fraction. In order to follow the nuclear accumulation of proteasomes, we generated and transiently overexpressed in PC3 cells the proteasomal subunit XAPC7 containing a C-terminal FLAG tag (Figures 5F and 5G). Exogenous XAPC7–FLAG has previously been used as a marker to observe the movement of proteasomes between the cytoplasm and the nucleus [36]. We also generated, and transiently over-expressed in PC3 cells, an XAPC7 variant containing a deletion of a NLS (XAPC7del), that has been shown to prevent stress-induced nuclear accumulation of proteasomes (dominant-negative effect) [36]. It is important to note that in the transient expression system, where an excessive level of exogenous protein is produced, only a small subpopulation of XAPC7 molecules would be expected to interact with the proteasomal complexes and therefore serve as a marker of proteasomal movement. The treatment of PC3 cells with MG132 caused the accumulation of both wtXAPC7 and XAPC7del in the nuclear-enriched fraction (Figure 5F), indicating that NLS deletion does not perturb the nuclear accumulation of proteasomes when induced by their direct inhibition. Clioquinol and copper treatment caused the concurrent accumulation of both XIAP and wtXAPC7 in the nuclear-enriched fraction (Figure 5G), analogous to MG132 treatment (Figure 5F). The same clioquinol and copper treatment also caused the accumulation of XAPC7del in the nuclear-enriched fraction (results not shown). Together these results demonstrate that both clioquinol treatment and conventional proteasomal inhibition (MG132 treatment) caused the concurrent accumulation of proteasomes and XIAP in the nuclear-enriched fraction. These results raise the intriguing possibility that clioquinol and copper causes cytosolic XIAP to traffic to the nucleus with proteasomes.

Figure 4 Cellular copper homoeostatic mechanisms influence sensitivity to clioquinol treatment

(A) Basal intracellular metal content of the prostate primary (PrEC), hyperplasic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cell lines were measured using inductively-coupled-plasma-MS. Results are the means ± S.D. (n = 4) and are shown as ng of metal per 10^6 cells. (B) PC3 cells transfected with pcDNA3, pcDNA3 encoding Ctr1–Myc, pEBB or pEBB encoding ATP7B–FLAG were analysed by Western blotting. ATP7B–FLAG, Ctr1–Myc, XIAP and β-actin were immunolabelled as described in the Experimental section. (C) PC3 cells transfected as shown in (B) were treated for 16 h with medium supplemented with clioquinol (10 μM) and CuCl2 (20 μM). For controls, untransfected cells were treated with medium supplemented with clioquinol (10 μM) and CuCl2 (20 μM), or with clioquinol (10 μM), CuCl2 (20 μM) and TTM (20 μM). Cell viability was determined by the propidium iodide dye-exclusion viability assay.

DISCUSSION

Avoidance of apoptosis is critical in the development and progression of cancer [38]. The metalloprotein XIAP is a potent anti-apoptotic protein that inhibits caspases and suppresses apoptosis at the final juncture, and is therefore an attractive therapeutic target for the treatment of malignancy. In the present study, we have demonstrated that the copper ionophore clioquinol rapidly diminishes not only cytosolic XIAP, but also the closely related cIAP1 and cIAP2 proteins (Figures 2, 3B and 3C) and selectively causes the death of transformed (hyperplasic and carcinoma) prostate cell lines (Figure 1C). Redistribution of cytosolic IAP family members, in particular XIAP, provides a mechanistic explanation for caspase activation upon clioquinol treatment. Their removal from the cytoplasm prevents direct inhibition of caspases, in particular caspases 3, 7 and 9, the enzymes primarily responsible for apoptotic cell death. Consistent with the selective nature of clioquinol toxicity, clioquinol treatment caused the redistribution of cytosolic XIAP in transformed prostate cells and not in primary PrECs, validating its therapeutic potential for prostate cancer.

XIAP has been shown to directly co-ordinate copper, but only when the cation exceeds normal physiological levels [28,29]. Pathological levels of copper, as found in hepatocytes of patients with Wilson disease, trigger reversible conformational changes in XIAP accelerating its degradation [29]. Copper can bind to the cysteine-rich BIR domains and RING finger, leading to a clear shift in the electrophoretic mobility of XIAP seen by Western blotting [29]. Despite demonstrating that clioquinol caused the profound accumulation of intracellular copper (Figure 3F), we
Clioquinol depletes cytoplasmic XIAP

Figure 5  XIAP domains undergo relocation to the nucleus via proteasomes

(A) Schematic representation of HA-tagged truncated versions of the XIAP orientated from the N- to C-terminus (left to right). Amino acid numbers are shown. (B) PC3 cells transfected with the indicated HA-tagged truncated XIAP construct were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl₂ (20 μM). Cytoplasmic preparations were analysed by Western blotting, and exogenous XIAP proteins and β-tubulin III were immunolabelled as described in the Experimental section. (C) PC3 cells transfected with the indicated HA-tagged truncated XIAP construct were treated for 6 h with either fresh medium (containing 0.2 % DMSO) or medium supplemented with both clioquinol (10 μM) and CuCl₂ (20 μM). Cytoplasmic (control) and nuclear-enriched fractions were analysed by Western blotting. Exogenous XIAP proteins and histone H2B were immunolabelled as described in the Experimental section. (D) PC3 cells were treated for 16 h with either fresh medium or medium supplemented with MG132 (10 μM). Cytoplasmic and nuclear-enriched fractions were analysed by Western blotting and endogenous XIAP and histone H2B were immunolabelled as described in the Experimental section. (E) PC3 cells were treated for 16 h with either fresh medium or medium supplemented with MG132 (10 μM). Total cellular lysates were analysed by Western blotting and endogenous XIAP, PTEN and β-actin were immunolabelled as described in the Experimental section. Values are means ± S.D. of quadruplicate determinations for each test condition. (F) PC3 cells transfected with pcDNA3, pcDNA3 encoding wtXAPC7, or pcDNA3 encoding XAPC7del were treated for 16 h with either fresh medium or medium supplemented with MG132 (10 μM). Nuclear-enriched fractions and a control cytoplasmic preparation (pcDNA3 transfected) were analysed by Western blotting. wtXAPC7, XAPC7del, XIAP, β-tubulin III and histone H2B were immunolabelled as described in the Experimental section. (G) PC3 cells transfected with either pcDNA3 or pcDNA3 encoding XAPC7 were treated for 6 h with either fresh medium (containing 0.2 % DMSO), or with medium supplemented with clioquinol (10 μM) and CuCl₂ (20 μM). Cytoplasmic- and nuclear-enriched fractions were analysed by Western blotting and wXAPC7, XIAP, β-tubulin III and histone H2B were immunolabelled as described in the Experimental section.

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did not observe any electrophoretic change in XIAP (Figure 2) and therefore saw no indication of direct copper binding. Other bivalent metals have also been shown to modulate cellular XIAP levels. Cadmium and the zinc-specific chelator TPEN [N,N,N’,N’-tetrakis-(2-pyridylmethyl)ethylendiamine] both depleted XIAP in PC3 cells and consequently sensitized the cells to TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-mediated apoptosis [39,40]. Both cadmium and TPEN possibly destabilize XIAP through a similar mechanism, by displacing structural zinc [39,40]. Our results indicate that clioquinol and copper may cause cytoplasmic XIAP to relocate to the nucleus as a result of proteasomal inhibition (Figures 5D–5G). We have demonstrated that the treatment of PC3 cells with either clioquinol or MG132 resulted in the redistribution of XIAP to the nuclear-enriched fraction (Figures 5D–5G). Cytoplasmic clearance of XIAP may therefore occur universally with proteasome inhibitors that specifically impede chymotrypsin-like activity [17,41], to allow for subsequent caspase activation.

Studies in humans revealed that serum levels of clioquinol could range from 13 to 25 μM when subjects are given a tolerated oral dose (125–375 mg) [42]. In our culture system, we established that 10 μM clioquinol was sufficient to rapidly kill multiple malignant prostate lines (less than 32 h at LD50) without affecting the viability of primary PrECs (Figure 1C). Several other groups have similarly found that clioquinol kills other types of malignant cells when at low concentrations [10–13]. We established that the selective toxicity of clioquinol was mediated in a dose-dependent manner by the level of extracellular copper (Figure 1B) and could be abrogated by removing bioavailable copper with high-affinity chelation (TTM treatment) (Figure 3C). Clioquinol treatment alone (no copper) was sufficient to kill PC3 cells, but only when administered at higher concentrations (>100 μM) (results not shown). In this instance, clioquinol alone may exploit the intracellular copper that accumulates in malignant cells to mediate its toxicity. Nevertheless, exogenous copper clearly and significantly enhances the toxicity of clioquinol when present at physiologically equivalent or greater concentrations (>20 μM). In addition, the key regulators of cellular copper homeostasis, ATP7B and Ctrl, were found to be influential factors in determining the degree of cellular sensitivity to clioquinol (Figure 4C). Both proteins have previously been shown to mediate the toxicity of other metal-based pharmaceuticals, including cisplatin [43,44], by directly regulating their intracellular level. Whether ATP7B and Ctrl directly interact with the clioquinol–copper complex is the subject of future investigations. Malignancy-driven changes in the expression profile and/or activity of the known cellular copper-binding molecules (transporters, chaperones, etc.) has not been reported. However, the marked difference in intracellular copper level and resultant oxidant pressure would conceivably necessitate numerous adaptive changes. These changes may underlie the reason for malignant cells being selectively sensitive to clioquinol (ionophoric copper) treatment.

Previous studies have also demonstrated that clioquinol toxicity can result from ionophoric-zinc uptake. Clioquinol with zinc causes lysosomal inhibition and caspase-mediated apoptosis in malignant cells [45]. In the cancer setting, it remains to be determined whether copper or zinc would be the more attractive target to aid clioquinol-induced malignant cell death. Clioquinol may favour copper metallation in the serum, as copper is significantly elevated in the serum of cancer patients, whereas the level of zinc is often reduced [6,7]. Even in healthy individuals, there is an approximately 3-fold more copper in the serum than zinc [copper (17–25 μM) compared with zinc (5–8 μM)], with the difference further widened by the malignant state. Clioquinol also has a greater affinity for copper ($K_i(\text{Cu}) = 8.9$ compared with $K_i(\text{Zn}) = 7.0$) [46] and encouragingly we have demonstrated that the toxicity of clioquinol towards transformed prostate cells positively correlates with the level of milieu copper (Figure 1B). Our results support the potential use of clioquinol as a therapeutic agent to treat prostate cancer. Comprehensive studies in animal models are required to address whether clioquinol administration can exploit elevated copper and induce selective malignant prostate cell death, as observed in vitro.

**AUTHOR CONTRIBUTION**

Michael A. Cater devised and executed all of the experiment and wrote the manuscript. Ygal Haupt provided financial support and the prostate experimental system, helped design the experiments, and edited the manuscript prior to submission.

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Clioquinol depletes cytoplasmic XIAP


SUPPLEMENTARY ONLINE DATA

Clioquinol induces cytoplasmic clearance of the X-linked inhibitor of apoptosis protein (XIAP): therapeutic indication for prostate cancer

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Figure S1 Clioquinol with copper induces the nuclear accumulation of XIAP

Immunofluorescent microscopy of endogenous XIAP in PC3 cells treated for 6 h with medium supplemented with clioquinol (10 μM) alone, CuCl₂ (20 μM) alone, or the combination of both clioquinol (10 μM) and CuCl₂ (20 μM). Detection of XIAP was performed using a mouse anti-XIAP antibody (dilution 1/50) followed by goat anti-mouse IgG antibody conjugated to Alexa Fluor® 488 (diluted 1/2000). The nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole 1pg/ml solution made in PBS) by incubating the cells for 5 min immediately following secondary antibody incubation. Photographs were taken using a 60 × water objective with an Olympus BX51 microscope.

Figure S2 Effect of clioquinol and copper on intracellular metal levels

PC3 cells were treated for 6 h with medium supplemented with CuCl₂ (20 μM) alone (containing 0.2 % DMSO) (Control), both clioquinol (10 μM) and CuCl₂ (20 μM), or both TTM (tetrathiomolybdate) (20 μM) and CuCl₂ (20 μM). Intracellular metal content was measured using inductively-coupled-plasma-MS. Results are the means ± S.D. (n = 4) and are shown as μg or ng of metal per 10⁶ cells.

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