



## Sodium arsenite down-regulates the expression of X-linked inhibitor of apoptosis protein via translational and post-translational mechanisms in hepatocellular carcinoma

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### ABSTRACT

X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitors of apoptosis protein (IAP) family, and has been reported to exhibit elevated expression levels in hepatocellular carcinoma (HCC) and promote cell survival, metastasis and tumor recurrence. Targeting XIAP has proven effective for the inhibition of cancer cell proliferation and restoration of cancer cell chemosensitivity. Arsenic (or sodium arsenite) is a potent anti-tumor agent used to treat patients with acute promyelocytic leukemia (APL). Additionally, arsenic induces cell growth inhibition, cell cycle arrest and apoptosis in human HCC cells. In this study, we identified XIAP as a target for sodium arsenite-induced cytotoxicity in HCC. The exposure of HCC cell lines to sodium arsenite resulted in inhibition of XIAP expression in both a dose- and time-dependent manner. Sodium arsenite blocked the *de novo* XIAP synthesis and the activity of its internal ribosome entry site (IRES) element. Moreover, treatment with sodium arsenite decreased the protein stability of XIAP and induced its ubiquitin–proteasomal degradation. Overexpression of XIAP attenuated the pro-apoptotic effect of sodium arsenite in HCC. Taken together, our data demonstrate that sodium arsenite suppresses XIAP expression via translational and post-translational mechanisms in HCC.

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### 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of death from cancer worldwide. Although HCC that is detected at an early stage can be managed with surgical resection or transplantation in select cases, advanced tumors respond poorly to currently available medical therapies [1]. Deregulation of the balance between cell proliferation and cell death is a common pro-tumorigenic theme in human hepatocarcinogenesis, which predominantly results from the over-activation of anti-apoptotic pathways [2]. Therefore, therapeutic strategies that selectively inhibit anti-apoptotic signals in liver tumor cells have the potential to become powerful tools for the treatment of HCC [2].

The inhibitors of apoptosis protein (IAP) represent a large family of endogenous caspase inhibitors [3]. XIAP is the most well-studied of the eight human IAPs and has been characterized as the most potent caspase inhibitor, functioning by directly binding to caspases through the baculovirus IAP repeat (BIR) domains [4]. The expression of XIAP is regulated at the level of translation. The

5'-untranslated region (UTR) of XIAP mRNA is reported to contain a 162-nucleotide (nt) internal ribosome entry site (IRES) sequence that is critical for the cap-independent translation of XIAP [5]. In addition, the C-terminal fragment of XIAP, which contains the BIR3 and RING finger domains, has been reported to be involved in the autoubiquitination and degradation of XIAP [6–8]. The XIAP BIR3 domain contains two autoubiquitination sites, Lys<sup>322</sup> and Lys<sup>328</sup> [9], whereas the RING domain possesses E3 ubiquitin ligase activity and is required for the autoubiquitination of XIAP [8,9]. Previous studies have demonstrated that XIAP expression is elevated in a variety of human tumors, including esophageal carcinoma [10], clear cell renal carcinoma [11], ovarian carcinoma [12] and lymphoma [13]. Furthermore, XIAP expression has been established as a risk factor in apoptosis resistance, invasiveness, metastasis, and tumor recurrence in HCC patients, suggesting that molecular targeting of XIAP has the potential to suppress HCC metastasis and recurrence [14].

Arsenic (or sodium arsenite) has been shown to be an effective inducer of cellular apoptosis in the clinical trials of acute promyelocytic leukemia (APL) patients; it is also approved by the US Food and Drug Administration for the treatment of relapsed APL that is refractory to therapy with all-trans retinoic acid (Food and Drug Administration (2000) FDA approves arsenic trioxide for leukemia treatment (<http://www.fda.gov/bbs/topics/ANSWERS/ANSO1040.html>)). Moreover, arsenic trioxide (or sodium arsenite) also exhibits therapeutic

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potential and antitumor activity in a variety of solid tumors including HCC [15–17]. Accumulating evidence indicates that arsenic treatment potently induces cell growth inhibition, cell cycle arrest and apoptosis in human HCC cells [18,19].

In this study, we examined whether XIAP was a target of sodium arsenite in HCC. Our data demonstrated that sodium arsenite down-regulated the expression of XIAP in HCC via translational and post-translational mechanisms. The observed decrease in XIAP translation may result from the inhibition of IRES element activity, whereas the post-translational regulation of XIAP results from ubiquitin–proteasomal degradation. Moreover, over-expression of XIAP impaired the pro-apoptotic effect of sodium arsenite in HCC.

## 2. Materials and methods

### 2.1. Antibodies and reagents

The rabbit anti-XIAP antibody was purchased from Cell Signaling Technology. The mouse anti-GAPDH antibody was purchased from Santa Cruz Biotechnology. Cycloheximide (CHX) was purchased from Sigma. The dual-luciferase reporter assay system was purchased from Promega.

### 2.2. Cell culture

Huh7 and BEL-7402 cell lines were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C with 5% CO<sub>2</sub>.

### 2.3. Construction of plasmids and transient cell transfection

The full-length amplified XIAP gene was inserted into the pEGFP-N3 vector to generate the pEGFP-XIAP plasmid. Transient transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

### 2.4. Dual-luciferase reporter gene assay

Dual-luciferase reporter gene assay (Promega) was performed according to the manufacturer's instructions, using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

### 2.5. [<sup>35</sup>S]-methionine incorporation assay

The cells were incubated with DMEM depleted of methionine for 30 min and then labeled with [<sup>35</sup>S]-methionine (Perkin Elmer). The cell extracts were subjected to immunoprecipitation with the XIAP antibody. The immunoprecipitates were eluted and applied to SDS–PAGE. Then, the gels were dried and visualized by phosphoimaging (Fujifilm).

### 2.6. Morphological analysis of apoptosis by fluorescent staining

Morphological analysis of apoptosis by fluorescent staining was performed according to our previous report [20]. Briefly, the cells grown on glass coverslips were fixed with 4% paraformaldehyde/PBS, permeabilized in 0.1% Triton X-100/PBS, and stained with Hoechst 33258. After the coverslips were washed in PBS, positive nuclei were counted. Normal nuclei and apoptotic nuclei (identified by their condensed new moon appearance or fragmented chromatin) were easily distinguished.

### 2.7. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. Sodium arsenite down-regulates the protein expression level of XIAP in HCC

XIAP functions as a potent suppressor of apoptosis by inhibiting the activities of caspase enzymes. Furthermore, XIAP has been reported to be elevated in HCC and promote cell survival, metastasis and tumor recurrence [14]. To determine whether XIAP is a target of sodium arsenite-induced cytotoxicity, we first examined the effect of sodium arsenite on XIAP expression in HCC cells (Huh7 and BEL-7402). As shown in Fig. 1A, the protein expression level of XIAP decreased in a dose-dependent manner when HCC cells were exposed to increasing concentrations of sodium arsenite. Moreover, sodium arsenite induced a rapid loss of XIAP protein as early as 1 h after treatment and promoted the decrease of XIAP expression in a time-dependent manner (Fig. 1B).

### 3.2. Sodium arsenite inhibits the *de novo* protein synthesis of XIAP and its IRES activity

To explore the mechanism by which sodium arsenite modulated the expression of XIAP, we first employed quantitative real-time PCR to determine whether the mRNA expression levels of XIAP were affected. As shown in Supplementary Fig. 1, treatment with sodium arsenite showed little effect on the mRNA transcript levels of XIAP, suggesting that sodium arsenite modulated the expression of XIAP at the post-transcriptional level.

To understand whether sodium arsenite modulated the expression of XIAP at the translational level, a [<sup>35</sup>S]-methionine incorporation assay was performed to detect the rate of *de novo* XIAP synthesis. As shown in Fig. 2A, treatment with sodium arsenite significantly suppressed the *de novo* protein level of XIAP. The effect of sodium arsenite on the XIAP IRES was also assessed using the bicistronic pCDNA/Fluc/IRES/Rluc reporter construct, which we described previously [21]. As shown in Fig. 2B, treatment with sodium arsenite significantly suppressed the XIAP IRES-mediated activity of the Renilla luciferase reporter in a time-dependent manner. Similar results were also observed in CHX-treated cells (Supplementary Fig. 2). However, treatment with PP242, a potent inhibitor of cap-dependent translation, showed little effect on the translation and expression of XIAP, suggesting that the protein synthesis of XIAP in HCC cells is IRES-mediated rather than cap-dependent (Supplementary Fig. 3). Taken together, these results indicate that sodium arsenite inhibits the translation of XIAP and that this inhibition may occur via suppression of XIAP IRES activity.

### 3.3. Sodium arsenite decreases the stability of XIAP and promotes its ubiquitin–proteasomal degradation

Evidence suggests that, in response to apoptotic stimuli, XIAP is capable of autoubiquitination, which leads to proteasomal degradation [7]. To determine whether proteasomal degradation was involved in the rapid loss of XIAP protein upon sodium arsenite treatment, the stability of XIAP was evaluated by cycloheximide chase. As shown in Fig. 3A, the turnover of XIAP protein was accelerated in cells treated with sodium arsenite (half-life of approximately 1 h) compared with control cells (half-life of greater than 1.5 h).

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