

Original Research Article

2-Amino-nicotinamide induces apoptosis of prostate cancer cells via inhibition of PI3K/AKT and phosphorylation of STA3/JAK2

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Sent for review: 31 October 2019

Revised accepted: 27 August 2020

Abstract

Purpose: To study the cytotoxicity of 2-amino-nicotinamide against prostate cancer (PCa) cells, and the underlying molecular mechanism.

Methods: The effect of 2-amino-nicotinamide on cell viability and apoptosis was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and flow cytometry, respectively, while its effect on cellular production of fluorescent-oxidized product from DCFH-DA was measured using flow cytometry. Apoptosis-related protein expressions were evaluated by western blot assay.

Results: 2-Amino-nicotinamide produced cytotoxicity against MCF-7, SGC7901, PCa 22Rv1 and LNCaP cancer cell lines ($p < 0.05$). Mechanistic data revealed that 2-amino-nicotinamide activated apoptosis, and enhanced cleavage of PARP and caspase-3 in PCa 22Rv1 and LNCaP cells. In PCa 22Rv1 and LNCaP cell lines, cytochrome C and Bax levels were enhanced by treatment with 2-amino-nicotinamide, while Bcl-2 protein level was suppressed ($p < 0.05$). Activated expressions of PI3K, Akt and ERK in PCa 22Rv1 and LNCaP cells were down-regulated, while p38 expression was increased. Moreover, 2-amino-nicotinamide suppressed the activation of JAK2 and STAT3, but did not alter total JAK2 and STAT3 levels in PCa 22Rv1 and LNCaP cells ($p < 0.05$).

Conclusion: 2-Amino-nicotinamide exerts cytotoxic effects on prostate carcinoma cells via activation of apoptosis and down-regulation of PI3K/AKT and STA3/JAK2. Thus, 2-amino nicotinamide is a potential bioactive agent for prostate cancer management.

Keywords: 2-Amino-nicotinamide, Apoptosis, Fluorescent-oxidized, Cytotoxicity

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INTRODUCTION

Prostate cancer is one of the common malignancies diagnosed in the genitourinary system of men, and it the second highest cause of cancer-related deaths in men in US [1]. In prostate cancer patients, morbidity has been

found to be related to family history, age factor and ethnicity [2]. The occurrence of prostate cancer and its progression are believed to involve interaction of genetic predisposition and environmental factors [3]. Susceptibility to prostate cancer is determined by genetic factors [3]. Analysis of the genomes of various ethnic

groups globally has led to identification of more than 30 susceptible sites which may be linked to the risk of prostate cancer [3].

Studies on the role of genetic predisposition in prostate cancer have led to remarkable results [4]. The proliferation of prostate cancer is independent of androgens, with gradual progression resulting in the development of resistance to drugs and secondary endocrine therapy [5]. High levels of serum interleukin-6 in prostate cancer cells lead to differentiation and transformation of malignant tumor cell to androgen-independent cells [6]. Interleukin-6 plays its role through various transducers and receptors such as p-Akt, p-ERK1/2 and Janus kinase (JAK). Moreover, signal transducer and activator of transcription (STAT) is used by interleukin-6 for transforming prostate cancer cells to androgen-independent form [6]. The main component which drives JAK/STAT pathway is p-STAT3. Many novel therapies have used JAK/STAT as the target for cancer treatment [7]. It has been reported that androgen receptor (AR) is activated by PI3K through interleukin-6, but the activation is not always dependent on PI3K pathway [8]. Apoptosis is stimulated by endogenous stress on cells [9]. The common stresses include aggregation of unfolded proteins in ER, calcium ion secretion from the ER and specific unprocessed proteins in the ER [9]. Apoptosis is generally induced by sustained stress in the endoplasmic reticulum [10]. The endoplasmic reticulum contains a member of pro-apoptotic protein family known as caspase-3 which is released during stress [11]. The present study was designed to investigate the cytotoxic effect of 2-amino-nicotinamide on prostate cancer cells, and the molecular mechanism involved.

EXPERIMENTAL

Drug and reagents

Nicotinamide derivative (2-amino-nicotinamide) was provided by Alfa Aesar (Thermo Fisher Scientific). Dimethyl sulfoxide (DMSO) and other reagents were obtained from Sigma-Aldrich.

Cell culture

The cancer cell lines MCF-7, SGC7901, PCa 22Rv1 and LNCaP were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in DMEM containing 10 % FBS, under humid atmosphere containing 5 % CO₂ at 37 °C.

MTT assay

At 80 % confluence, MCF-7, SGC7901, PCa 22Rv1 and LNCaP cancer cells were plated in 96-well plates, each at a density of 2x10⁶ cells per well. The cells were incubated in DMEM containing 10 % FBS for 24 h, followed by replacement of the culture medium with fresh medium containing graded concentrations of 2-amino nicotinamide i.e. 5, 10, 20, 40, 80, 160 and 320 μM for 72 h. At 24, 48 and 72 h of incubation, 25 μL MTT solution was added to cells in each well, and incubation was continued for 4 h. Then, the medium in each well was replaced with DMSO (150 μl) to dissolve the solid formazan crystals formed. The optical density of each well was read at 568 nm in Varioskan Flash Multimode Reader.

Apoptosis assay

The PCa 22Rv1 and LNCaP carcinoma cells at 80 % confluence were dispersed in glass cover slips of Lab-Tek Chamber Slides at a density of 2 x 10⁶ cells per well. The cells were incubated with 2-amino nicotinamide at concentrations of 10, 20 and 40 μM for 72 h. Cells exposed to RQ1 RNase-Free DNase (one unit/100 μL) served as control. Thereafter, the cells were fixed for 35 min in 4 % paraformaldehyde, after which they were stained with fluorometric TUNEL system (Promega, Madison, WI, USA) in accordance with the manufacturer's procedure. The experiments were conducted in triplicate.

Detection of ROS

In PCa 22Rv1 and LNCaP cells (2 x 10⁵ cells per well) exposed to 2-amino nicotinamide, ROS levels were determined using 2,7-dichlorofluorescein diacetate (DCFH-DA) stain. The cells were exposed 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h. Then, the cells were centrifuged, washed three times with PBS, and treated with 10 μM DCFH-DA. After incubation under darkness for 20 min, the cells were washed with PBS. Subsequently, fluorescent-oxidized product formed from DCFH-DA was monitored using flow cytometer (Beckman Coulter Inc.).

Western blot assay

In this assay, PCa 22Rv1 and LNCaP cells were treated for 72 h with 2-amino nicotinamide at doses of 10, 20 and 40 μM in 6-well plates, each cell line at a density of 2x10⁶ cells per well. The cells were washed twice in cold PBS and subsequently lysed with ProteoJET cytoplasmic extraction kit (Fermentas; Thermo Fisher

Scientific, Inc.). The protein contents of the lysates were determined using BCA Protein Assay kit (Thermo Fisher Scientific Inc.). Then, 30- μ g protein samples were separated on 12 % SDS-PAGE, followed by electrophoretic transfer to PVDF membranes. The membranes were blocked with 5 % non-fat milk in TBST, prior to overnight incubation with primary antibodies at 4°C. The antibodies used were anti-p-PI3 kinase, anti p-Akt, anti-JAK2, anti-p-Jak2, anti-p-ERK, anti-p38, anti-STAT3, anti-p-STAT3, anti-Bcl-2 and anti-Bax (Cell Signaling Technology, Inc. Danvers, MA, USA). Then, the membranes were rinsed with PBS, followed by incubation with horseradish peroxidase-conjugated rabbit secondary antibody for 1.5 h at room temperature. Enhanced chemiluminescence (ECL) development reagent was used for detection of the blots, while sodium Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.) was used for blot analysis.

RT-qPCR assay

Total RNA was extracted from PCa 22Rv1 and LNCaP cells using TRIZOL reagent (Life Technologies; Thermo Fisher Scientific, Inc.) in line with the manual protocols. Then, 1 μ g RNA from each sample was reverse-transcribed to first-stand cDNA using the Super-Script First-Strand cDNA System (Invitrogen). Signal amplification was made using Platinum SYBR-Green qPCR Super Mix-UDG (Invitrogen). The master mix in each PCR reaction consisted of qPCR Super Mix-UDG, cDNA template, as well as forward and backward primers. The conditions used were: 15 min at 93 °C, then 50 cycles at 93 °C for 25 sec, 58°C for 50 min and 70 °C for 25 sec. The relative gene expression levels were normalized to that of β -actin.

Statistical analysis

Data are presented as mean \pm standard deviation (SD) of triplicate values. Statistical analysis of data was made with SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA). Data were compared using one-way analysis of variance, followed by Tukey's post-hoc test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Cytotoxic effect of 2-amino nicotinamide on lung, gastric and prostate cancer cells

As shown in Figure 1, 2-amino nicotinamide significantly and dose-dependently inhibited the

growth of the tested cancer cells ($p < 0.05$). The half-maximal inhibitory concentration (IC_{50}) values of 2-amino nicotinamide for MCF-7, SGC7901, PCa 22Rv1 and LNCaP cells were 80, 40, 20 and 20 μ M, respectively. Thus, prostate carcinoma cells were more sensitive to 2-amino nicotinamide than lung and gastric cancer cells.

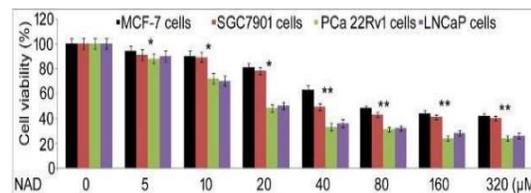


Figure 1: Effect of 2-amino nicotinamide on lung, gastric and prostate cancer cells. The MCF7, SGC7901, PCa 22Rv1 and LNCaP cells were exposed to 2-amino nicotinamide at doses of 5, 10, 20, 40, 80, 160 and 320 μ M. Cell viability measurements were made using MTT assay at 72 h of incubation; * $p < 0.05$; ** $p < 0.02$, vs. control

2-Amino nicotinamide inhibited viability of PCa 22Rv1 and LNCaP cells

The inhibition of viabilities of PCa 22Rv1 and LNCaP cell by 2-amino nicotinamide was dependent on duration of treatment (Figure 2). The IC_{50} values of 2-amino nicotinamide for PCa 22Rv1 cells at 24, 48 and 72 h were 80, 40 and 20 μ M, respectively. Treatment of LNCaP cells with 2-amino nicotinamide for 24, 48 and 72 h resulted in IC_{50} values of 160, 40 and 20 μ M, respectively. Therefore, in subsequent experiments, the cells were treated with 2-amino nicotinamide at doses of 10, 20 and 40 μ M for 72 h.

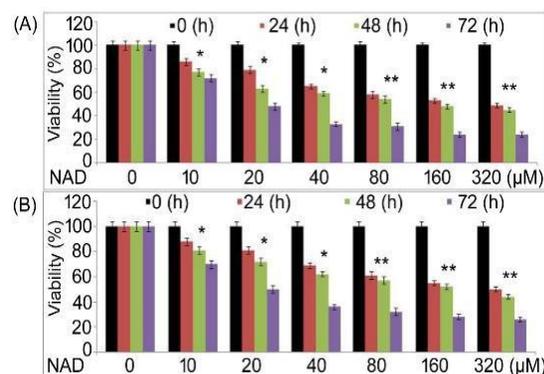


Figure 2: Effect of 2-amino nicotinamide on prostate cancer cells. PCa 22Rv1 cells (A) and LNCaP cells (B) were exposed to 2-amino nicotinamide at doses of 5, 10, 20, 40, 80, 160 and 320 μ M for 24, 48 and 72 h. Cell viability measurements were made using MTT assay. * $P < 0.05$; ** $p < 0.02$, vs. control

2-Amino nicotinamide induced apoptosis in PCa 22Rv1 and LNCaP cells

Results from flow cytometry showed that exposure of PCa 22Rv1 cells to 2-amino nicotinamide at doses of 10, 20 and 40 μM resulted in 23.42, 53.67 and 93.82 % apoptotic cells, respectively. In LNCaP cells exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h, the apoptotic cells were 19.67, 46.92 and 89.65 %, respectively.

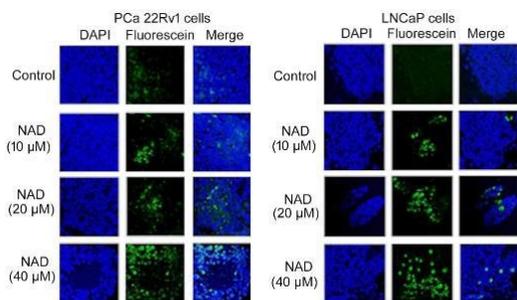


Figure 3: Effect of 2-amino nicotinamide on apoptosis of PCa 22Rv1 and LNCaP cells. The cells were exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM , or DMSO, and were stained using TUNEL staining for flow cytometric analysis. * $P < 0.05$; ** $p < 0.01$, vs. control

2-Amino nicotinamide enhanced cleavage of PARP and caspase-3 in PCa 22Rv1 and LNCaP cells

Treatment of PCa 22Rv1 and LNCaP cells with 2-amino nicotinamide at doses of 10, 20 and 40 μM promoted the cleavage of poly ADP-ribose (PARP) and caspase-3 (Figure 4).

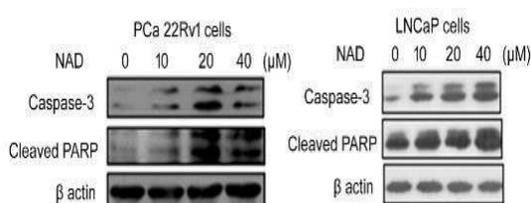


Figure 4: Effect of 2-amino nicotinamide on cleavage of PARP and caspase-3 in PCa 22Rv1 and LNCaP cells. (A) The cells were exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM , or DMSO for 72 h. Then, western blotting was used to determine the expressions of PARP and caspase-3, with β -actin as control

Nicotinamide derivative enhanced ROS generation in PCa 22Rv1 and LNCaP cells

Treatment of PCa 22Rv1 cells with 2-amino nicotinamide at doses of 10, 20 and 40 μM increased ROS levels to 41.64 ± 3.21 , $68.75 \pm$

3.90 and 86.82 ± 3.43 %, respectively, relative to 14.36 ± 1.28 % in untreated cells. The levels of ROS in LNCaP cells increased to 39.32 ± 2.14 , 64.76 ± 2.87 and 84.98 ± 3.93 % on exposure to 2-amino nicotinamide at doses of 10, 20 and 40 μM , respectively, relative to 13.56 ± 1.09 % in control.

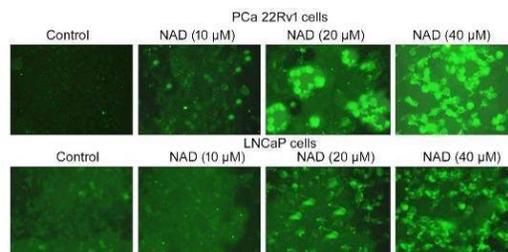


Figure 5: Effect of 2-amino nicotinamide on ROS production. PCa 22Rv1 and LNCaP cells were exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h. The ROS generation was measured with flow cytometry after DCFH-DA staining

2-Amino nicotinamide altered protein expressions in PCa 22Rv1 and LNCaP cells

Western blotting was used to determine changes in cytochrome c, Bax and Bcl-2 due to treatment of PCa 22Rv1 and LNCaP cells with 2-amino nicotinamide for 72 h. The treatment markedly promoted cytochrome c release into the cytoplasm in PCa 22Rv1 and LNCaP cells (Figure 6). The levels of Bax in PCa 22Rv1 and LNCaP cells were also increased on treatment with 2-amino nicotinamide at doses of 10, 20 and 40 μM . However, Bcl-2 expression was markedly decreased in these cells.

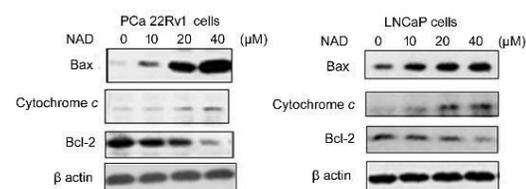


Figure 6: Effect of 2-amino nicotinamide on protein expressions of Bax, Bcl-2 and cytochrome c in PCa 22Rv1 and LNCaP cells exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h. The Bax, Bcl-2 and cytochrome c levels were determined with western blotting, with β -actin as control

Nicotinamide derivative activated p38 and deactivated PI3K/Akt/ERK in PCa 22Rv1 and LNCaP cells

In PCa 22Rv1 and LNCaP cells, p38 expression was markedly and concentration-dependently elevated on exposure to 2-amino nicotinamide

at doses of 10, 20 and 40 μM for 72 h (Figure 7). In contrast, the expressions of PI3K, Akt and ERK were significantly suppressed in PCa 22Rv1 and LNCaP cells by the same treatments.

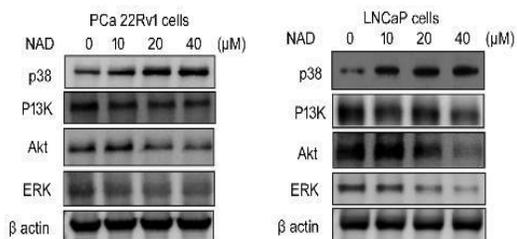


Figure 7: Effect of 2-amino nicotinamide on p38/PI3K/Akt/ERK. The expressions of activated p38, PI3K, Akt and ERK were determined in PCa 22Rv1 and LNCaP cells using western blot assay, following treatment of the cells with 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h

Nicotinamide derivative suppressed STAT3 phosphorylation in PCa 22Rv1 and LNCaP cells

The expressions of JAK2 and STAT3 were reduced in PCa 22Rv1 and LNCaP cells on treatment with 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h (Figure 8). However, 2-amino nicotinamide had no effect on the expressions of total JAK2 and STAT3 in PCa 22Rv1 and LNCaP cells.

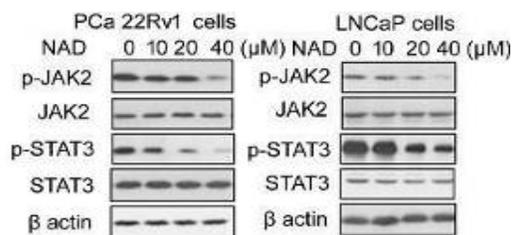


Figure 8: Effect of 2-amino nicotinamide on expressions of JAK2 and STAT3. The expression levels of JAK2, STAT3, p-JAK2 and p-STAT3 in PCa 22Rv1 and LNCaP cells exposed to 2-amino nicotinamide at doses of 10, 20 and 40 μM for 72 h were assayed with western blotting, with β -actin as internal control

DISCUSSION

Overproduction of ROS in carcinoma cells is involved in activation of cellular apoptosis via the mitochondrial pathway [12]. In many types of carcinoma cells (including prostate), ROS production results in the collapse of mitochondrial membrane potential and apoptosis [13]. In many cancer cells such as leukemia and melanoma, excessive generation

of ROS and destabilization of mitochondrial membrane effectively induce apoptosis [14]. Reactive oxygen species (ROS) are considered to be indicators of cellular oxidative stress and initiators of apoptosis via the mitochondrial pathway [15]. The intrinsic apoptotic pathway is characterized by general collapse of MMP, followed by membrane permeabilization and subsequent pro-apoptotic protein release [16].

Many of the potent anti-cancer compounds such as betulinic acid and vitamin E exhibit their effects by targeting the mitochondria, thereby increasing ROS levels [17]. In the current study, the cytotoxicity of 2-amino nicotinamide was initially determined against MCF-7 (lung), SGC7901 (gastric), and PCa 22Rv1 and LNCaP (prostate) cancer cell lines. The data clearly showed that 2-amino nicotinamide was cytotoxic against the four tested cancer cell lines, with maximum viability inhibitory effect against prostate cancers cells. The mechanism underlying the cytotoxicity of 2-amino nicotinamide was studied against PCa 22Rv1 and LNCaP carcinoma cells. TUNEL staining assay revealed that 2-amino nicotinamide-induced inhibition of the viabilities of PCa 22Rv1 and LNCaP cells was associated with activation of apoptosis. To confirm whether PCa 22Rv1 and LNCaP cell apoptosis was accompanied by overproduction of ROS, DCFH-DA stained cells were subjected to flow cytometry, and it was observed that exposure of PCa 22Rv1 and LNCaP cells to 2-amino nicotinamide resulted in excessive generation of ROS, when compared with control cells.

Moreover, 2-amino nicotinamide promoted the release of cytochrome c into the cytosol, and increased levels of Bax in PCa 22Rv1 and LNCaP cells. In addition, it suppressed the levels of the anti-apoptotic factor, Bcl-2 in PCa 22Rv1 and LNCaP cells. Thus, ROS overproduction was responsible for the cytotoxic potential of 2-amino nicotinamide against PCa 22Rv1 and LNCaP cells. This finding is consistent with previous reports. Cancer cell growth and apoptosis are regulated by the NF- κ B pathway, and this has led to the identification of several functional components such as PI3K/Akt, ERK and p38 [18,19]. Suppression of activation of STAT3 and JAK2 through phosphorylation has been found to activate carcinoma cell apoptosis [20].

In the present study, 2-amino nicotinamide also targeted STAT3 and JAK2 activation in PCa 22Rv1 and LNCaP cells. There was increase in p38 expression, and suppression in expression levels of PI3K, Akt and ERK in PCa 22Rv1 and

LNCaP cells treated with 2-amino nicotinamide. Studies have shown higher expression levels of STAT3 and Akt in carcinoma cells possessing anti-apoptotic potential [30]. Down-regulation of PI3K inhibits Akt which subsequently targets STAT3 [21]. Moreover, Akt down-regulation via suppression of JAK2 leads to inhibition of STAT3 [22]. The regulation of JAK2 is reduced on increasing Akt, which then reduces Akt and STAT3, indicating a negative feedback relationship [23]. In the present study, treatment of PCa 22Rv1 and LNCaP cells with 2-amino nicotinamide enhanced p38 expression and markedly inhibited activation of PI3K, Akt and ERK. In addition, JAK2 and STAT3 activation was also inhibited in PCa 22Rv1 and LNCaP cells by 2-amino nicotinamide.

CONCLUSION

These results indicate that 2-amino nicotinamide exhibits cytotoxic effect against prostate carcinoma cells via a mechanism involving apoptosis induction, up-regulation of ROS and up-regulation of the expressions of apoptotic factors. Moreover, 2-amino nicotinamide suppresses the activation of PI3K/AKT and STA3/JAK2 pathway. Therefore, 2-amino nicotinamide is a potential drug for the treatment of prostate cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Junhui Ying designed the study and wrote the paper. Changchun Zhou, Yili Jin, Daqiao Lu, Bing Xiong and Jiahui Wei performed the experimental work. Changchun Zhou and Yili Jin carried out the literature study and compiled the data. Daqiao Lu, Bing Xiong and Jiahui Wei performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors before communication for the consideration of publication.

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