NB1011 induces Ser15 phosphorylation of p53 and activates the G_2/M checkpoint

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NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, is a novel anti-cancer agent that selectively targets tumor cells expressing high levels of thymidylate synthase (TS), an enzyme required for DNA biosynthesis. NB1011 treatment of high-TS-expressing breast carcinoma cells (MCF7TDX) results in the induction of p53 and p21 protein levels, whereas no p53 or p21 induction is observed in the low-TS-expressing MCF7 tumor cells. Furthermore, MCF7TDX cells accumulate in the G_2/M phase of the cell cycle in response to NB1011. In this study, the effect of NB1011 on the phosphorylation status of p53 was analyzed. We demonstrate that NB1011 treatment of various tumor cell lines expressing high TS results in the phosphorylation of p53 on Ser15, whereas this p53 phosphorylation is not observed in low-TS-expressing tumor cells. Also, we examined the role of several key cell cycle regulators in the growth inhibition observed in response to NB1011. Our results show that the mRNA and protein levels of the G_2/M regulators cdc2, cyclin B1 and cdc25C are down-regulated in MCF7TDX cells, while unaffected in MCF7 cells. The mRNA and protein levels of 14-3-3σ, also a direct transcriptional target of p53, are up-regulated in MCF7TDX cells following NB1011 treatment, while unchanged in MCF7 cells. Taken together, our data indicate that the growth inhibition caused by NB1011 in MCF7TDX cells is mediated through phosphorylation of p53 and activation of the G_2/M checkpoint. *Anti-Cancer Drugs* 14:449–455 © 2003 Lippincott Williams & Wilkins.

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Introduction

Thymidylate synthase (TS) is the rate-limiting enzyme in the *de novo* synthesis of thymidylate. TS catalyzes the reductive methylation of dUMP to dTMP and thus is essential to cellular proliferation, which makes it a valuable chemotherapeutic target [1]. Several anti-cancer drugs, including 5-fluorouracil (5-FU) and Tomudex, function by inhibiting TS catalytic activity.

However, efforts to treat cancer by inhibiting TS face the common problem of resistance. Many cancer cells contain elevated levels of TS due to loss of tumor suppressor gene function, such as p53 or Rb. These increased TS levels render them less sensitive to TS inhibitors than normal cells [2,3]. Furthermore, evidence of intratumoral TS gene amplification as a mechanism of resistance to the TS inhibitors Tomudex and 5-FU has been well documented *in vitro*, using tumor cell lines, as well as *in vivo*, using tumor samples from patients [4–6]. Importantly, the significantly higher level of TS protein correlates with more rapidly progressing disease and poor prognosis [7–10].

Enzyme catalyzed therapeutic activation (ECTA) is an approach to drug development that exploits enzymes which are specifically overexpressed in cancer cells either intrinsically or due to drug resistance. NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, was developed using this ECTA approach. NB1011 is preferentially cytotoxic to tumor cells overexpressing TS [11]. NB1011 has also been shown to be efficacious in mouse xenograft models against tumors with high TS levels [12]. Furthermore, tumor cells overexpressing TS were shown to accumulate in the G_2/M phase of the cell cycle in the presence of NB1011, suggesting that a G_2/M checkpoint might be activated [12].

The G_2/M checkpoint exists to ensure that mitosis does not occur in the presence of DNA damage. Failure of this checkpoint function can lead to genomic instability and uncontrolled proliferation, a hallmark of cancer. When DNA damage occurs the tumor suppressor p53 becomes activated. One of the major mechanisms of p53 activation is through stabilizing phosphorylations in the N-terminus. Once active, p53 halts cell growth by inducing cell cycle arrest and/or apoptosis. The cellular response is dependent on several factors in the cell that influence which target genes are induced and repressed by p53 [13]. Induction of p21, GADD45 and 14-3-3σ by p53 has been shown to be involved in the activation of the G_2/M checkpoint [14–16]. All three of these gene products

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inhibit cdc2, the kinase responsible for entry into mitosis [17]. Moreover, activation of p53 leads to the down-regulation of cdc2 levels as well as the levels of cyclin B1, the regulatory subunit required for cdc2 kinase activity [18,19]. To elucidate further the mechanism by which NB1011 suppresses cellular growth we examined the phosphorylation status of p53 and the response of several key G_2/M checkpoint regulators to treatment of NB1011.

Methods

Cell culture

The breast adenocarcinoma cell line MCF7 and the colon adenocarcinoma cell line RKO were obtained from ATCC (Rockville, MD). The Tomudex-resistant breast cancer cell line MCF7TDX [20] and the Tomudex-resistant colon cancer cell line RKOTDX were provided by Dr P. Johnston (Queens University, Belfast, UK). Cells were cultured in RPMI supplemented with 10% fetal bovine serum and the antibiotic/antimycotic Fungizone (Gibco, Grand Island, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. MCF7TDX and RKOTDX were continuously cultured with 2 μM Tomudex to maintain resistance.

cDNA expression array

MCF7TDX cells were left untreated or treated with 30 μM NB1011 (this concentration is 10 times the IC_{50} for NB1011 to ensure that the majority of cells would be affected) for 48 h. Cells were trypsinized, spun down and frozen using liquid nitrogen. The cell pellets were subjected to cDNA expression analysis by Clontech (Palo Alto, CA) using the AtlasPure Total RNA Labeling System, the Atlas cDNA Expression Array and analyzed with Atlas Image 2.0 software (Clontech).

Western blot analysis

Cells were seeded in 75-cm^3 flasks at 5×10^5 cells per flask and incubated overnight. Cells were then treated with 30 μM NB1011 or left untreated. Following 24, 48 or 72 h of incubation cells were trypsinized and subsequently lysed at 4°C in lysis buffer containing 50 mM HEPES, 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Lysates were centrifuged at 4°C to pellet and remove insoluble material. Protein concentrations were then determined using BCA (Pierce, Rockford, IL). Protein lysates (30 or 100 μg) were resolved on SDS–PAGE gels (Novex, San Diego, CA). Samples were transferred to PVDF membrane (Pell Gelman, Ann Arbor, MI) and blocked in Blotto [5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST; Sigma, St Louis, MO)] for 2 h. The membrane was incubated with the primary antisera (diluted in Blotto) for 2 h at room temperature. Membranes were washed (4 times for 15 min each) in TBST and then incubated with the horseradish peroxidase-coupled secondary antisera (Amersham Life Science, Piscataway, NJ) for 1 h at room temperature. After four additional 15-min washes with TBST, the bands were visualized with the ECL detection system (Pierce,). Tubulin or β-actin protein levels were also determined and served as an internal control to assure equivalent levels of total lysate in each sample. Quantitative analysis of Western blots was performed on a Storm Phosphorimager. The following primary antibodies were used: cdc2 p34 sc-54 (Santa Cruz Biotechnology, Santa Cruz, CA), cdc25C sc-327 (Santa Cruz Biotechnology), cyclin B1 Ab-3 (NeoMarkers, Freemont, CA), p53 (DO-1) sc-126 (Santa Cruz Biotechnology), phospho-p53 (Ser15) rabbit polyclonal (Cell Signaling, Beverly, MA), 14-3-3σ Ab-1 (NeoMarkers), β-actin (Sigma) and tubulin Ab-4 (NeoMarkers).

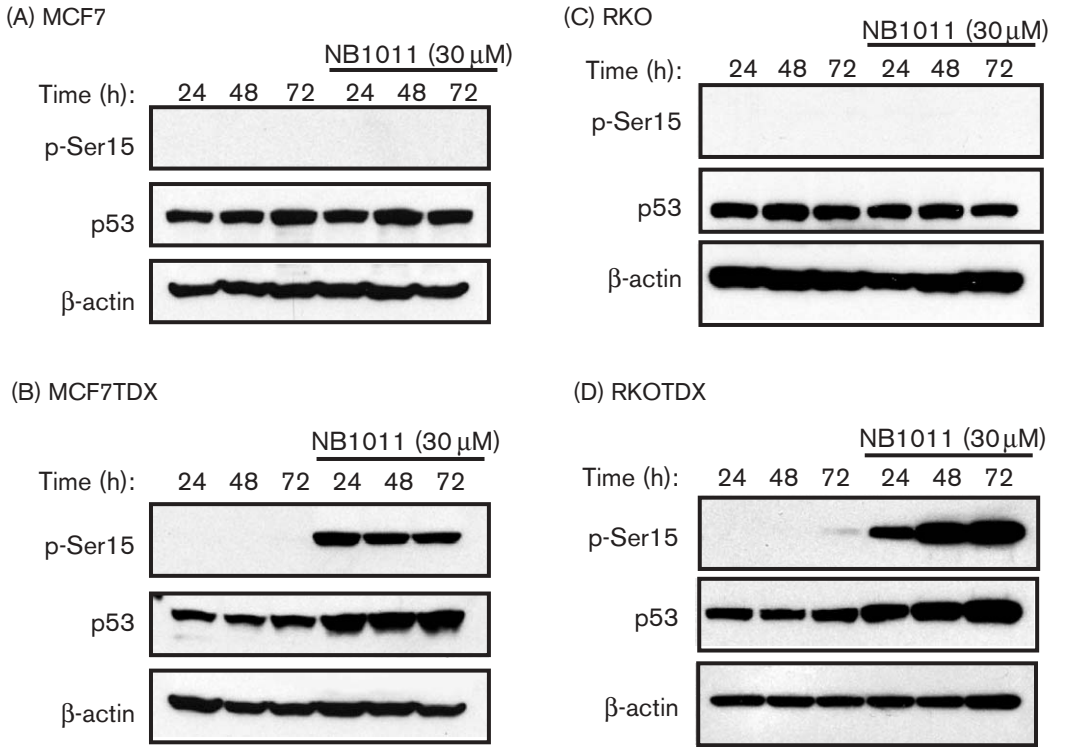
Results

NB1011 treatment of high-TS-expressing cells results in the phosphorylation of p53 on Ser15

Genotoxic stress activates p53, which subsequently directs the expression and repression of its downstream target genes, resulting either in a G_0/G_1 or G_2/M cell cycle arrest, to allow for repair of DNA damage, or in the induction of apoptosis. An essential step in the activation of p53 is the phosphorylation of the N-terminal region, which stabilizes p53 and stimulates binding of p53 to specific DNA sequences. Specifically, the p53 residue Ser15 has been reported to be directly phosphorylated by the Ser/Thr kinase ATM, which is rapidly activated upon DNA damage [21–24].

NB1011 is preferentially cytotoxic to tumor cells containing elevated TS protein levels [11]. Previously, we demonstrated that NB1011 induces p53 protein levels in the high-TS-expressing Tomudex-resistant MCF7TDX breast carcinoma cells, while this induction was not observed in the low-TS-expressing MCF7 cells [12]. To elucidate further the role of p53 in NB1011-induced growth inhibition, we examined the phosphorylation state of p53 at Ser15 in response to NB1011. Two high-TS-expressing cell lines (MCF7TDX and RKOTDX) and two low-TS-expressing cell lines (MCF7 and RKO) were examined at 24, 48, and 72 h in the presence or absence of NB1011. Lysates from these different conditions were analyzed for Ser15 phosphorylation of p53, total p53 and β-actin levels by Western blot. In low-TS-expressing cells, NB1011 treatment does not affect p53 levels (Fig. 1A and C, middle panel) and no Ser15 phosphorylation could be detected (Fig. 1A and C, upper panel). In contrast, p53 levels increase in response to NB1011 in high-TS-expressing MCF7TDX and RKOTDX cells (Fig. 1B and D, middle panel, respectively). Induction of Ser15 phosphorylation was observed following treatment with

Fig. 1



NB1011 increases p53 protein levels and induces the phosphorylation of p53 on Ser15 in cancer cell lines expressing high levels of TS. The tumor cell lines MCF7 (A) and RKO (C), which both express low levels of TS, and two tumor cell lines expressing high levels of TS, MCF7TDX (B) and RKOTDX (D), were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (100 μg) were examined for total p53 expression (middle panel), Ser15 phosphorylation of p53 (upper panel) and β-actin (lower panel).

NB1011 in both of these cell lines (Fig. 1B and D, upper panel).

The expression of several key G_2/M checkpoint regulators is altered in response to NB1011

To elucidate the effect of NB1011 on gene expression in more detail, cDNA expression analysis (Clontech) was performed using MCF7TDX cells treated with 30 μM NB1011 for 48 h or left untreated. Results from this analysis (Table 1) show clear changes in the expression levels of several cell cycle regulators involved in the G_2/M checkpoint. Specifically, three key positive regulators of the G_2/M transition, cdc2, cyclin B1 and cdc25C were found to be down-regulated in response to NB1011. Conversely, three key negative regulators of the G_2/M transition, p21, 14-3-3σ and GADD45, were found to be up-regulated in response to NB1011. The transcription of these six regulatory genes can be directly altered by p53. Binding of activated p53 to the promoters of p21, 14-3-3σ and GADD45 elicits their expression [14–16], while the transcription of cdc2, cyclin B1 and cdc25C is repressed by activated p53 [18,19,25]. The gene expression profile induced in MCF7TDX cells upon NB1011 treatment is consistent with the regulation of p53 target genes that result in a G_2/M cell cycle arrest. These results may therefore provide the molecular mechanism for the reported accumulation of MCF7TDX cells in the G_2/M phase of the cell cycle in response to NB1011 [12].

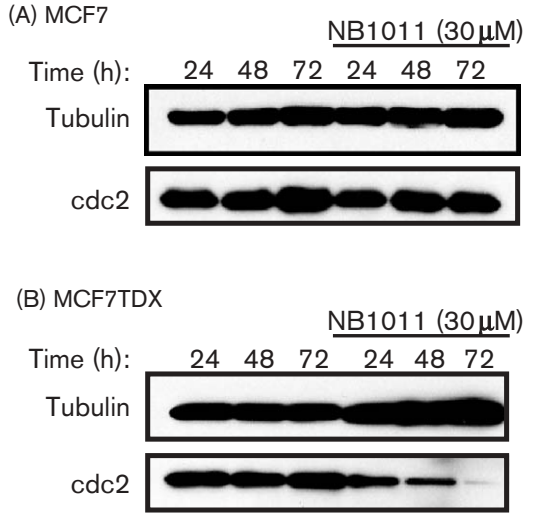
Table 1 Alterations in the mRNA expression of several key G_2/M cell cycle regulators in response to NB1011 in MCF7TDX cells (cDNA expression analysis was performed on MCF7TDX cells treated with 30 μM NB1011 for 48 h and compared to untreated cells)

	Fold
Down-regulated	
cdc2	3.0
cyclin B1	2.5
cdc25C	1.9
Up-regulated	
p21	11.8
14-3-3σ	3.1
GADD45	4.5

NB1011 down-regulates cdc2 and cyclin B1 protein levels in MCF7TDX cells, but not in MCF7 cells

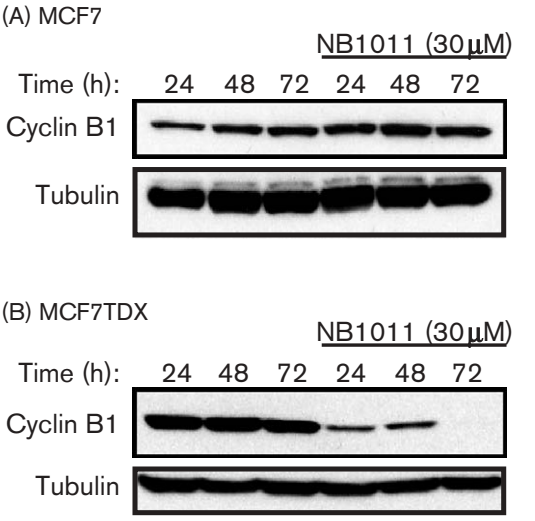
Cdc2 (also known as cdk1) is recognized as the enzyme that is responsible for entry into mitosis [26–28]. As such, cdc2 is very tightly regulated in a variety of ways including transcription, localization, phosphorylation and association with its regulatory subunit cyclin B1, whose

Fig. 2



NB1011 down-regulates cdc2. (A) MCF7 cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for cdc2 expression (lower panel) and tubulin (upper panel). (B) MCF7TDX cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for cdc2 expression (lower panel) and tubulin (upper panel).

Fig. 3



NB1011 down-regulates cyclin B1. (A) MCF7 cells were left untreated or treated with 30 μM NB1011 for 24, 48 or 72 h. Cell lysates (30 μg) were examined for cyclin B1 expression (upper panel) and tubulin (lower panel). (B) MCF7TDX cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for cyclin B1 expression (upper panel) and tubulin (lower panel).

expression is also tightly regulated. In order for mitosis to occur, cdc2 forms a complex with cyclin B1. Next, the two inhibitory phosphorylations of cdc2, at Thr14 and Tyr15, are removed by the dual specificity phosphatase, cdc25C. Finally, the cdk-activating kinase (CAK) activates cdc2 by phosphorylation at Thr161 [26,28,29]. The cdc2/cyclin B1 complex has been reported to be the main target for p53 regulation of the G_2/M transition. Activated p53 uses various mechanisms to inhibit cdc2/cyclin B1 kinase activity, including direct repression of cdc2 and cyclin B1 expression [18,19]. Since NB1011 elicits p53 phosphorylation and a decrease in the mRNA levels of cdc2 and cyclin B1, we examined the corresponding protein levels by Western blot analysis.

MCF7 and MCF7TDX cells were treated with 30 μM NB1011 or left untreated. Cells were collected at 24, 48 and 72 h after treatment and lysed. Lysates were analyzed for cdc2 (Fig. 2) or cyclin B1 (Fig. 3) protein levels. As shown in Fig. 2(B), cdc2 protein levels decrease in response to NB1011 in MCF7TDX cells and at 72 h the cdc2 protein level is almost below the level of detection. In contrast, NB1011 treatment of MCF7 cells does not result in a decrease of cdc2 protein levels (Fig. 2A). A similar trend was observed for cyclin B1 expression (Fig. 3). Cyclin B1 levels decrease after NB1011 treatment and are below detection at 72 h in MCF7TDX cells, while the levels remain consistent in MCF7 cells.

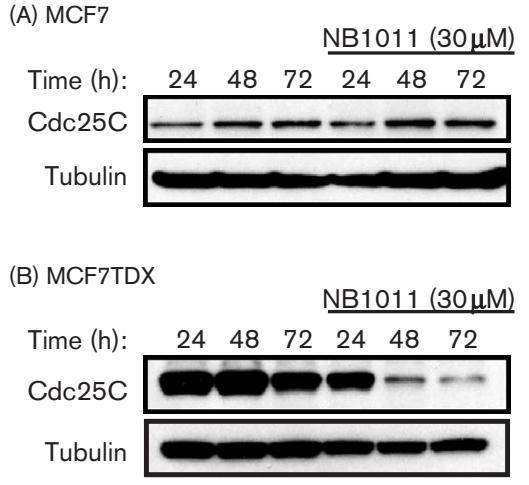
NB1011 decreases cdc25C protein levels in MCF7TDX cells, but not in MCF7 cells

Cdc25C is responsible for removing the inhibitory phosphorylations on cdc2 [30]. Thus, cdc25C is a positive regulator of G_2/M progression. Like cdc2, cdc25C activity is regulated by expression, phosphorylation and localization. When cdc25C becomes phosphorylated on Ser216, 14-3-3 proteins are able to bind directly to cdc25C and render it inactive through complex formation and localization to the cytoplasm [31,32]. Furthermore, cdc25C transcription is repressed when p53 is activated [25]. Cdc25C protein levels were examined by Western blot using MCF7 and MCF7TDX cells. As shown in Fig. 4(B), NB1011 treatment of MCF7TDX cells results in the reduction of cdc25C protein levels. In contrast, the levels of cdc25C remained consistent in MCF7 cells upon treatment with NB1011 (Fig. 4A).

NB1011 increases 14-3-3σ protein levels

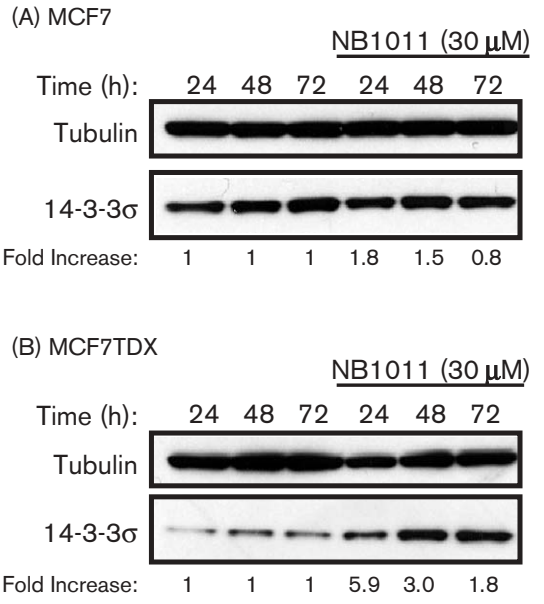
When p53 becomes activated, 14-3-3σ protein levels are up-regulated [16]. 14-3-3σ has been reported to bind to the cdc2/cyclin B1 complex and sequester it in the cytoplasm, rendering it inactive, and prevent progression of the cell cycle [17]. Since the mRNA levels of 14-3-3σ were found to be elevated after treatment with NB1011 (Fig. 1), protein levels were examined under these same conditions by western blot. As seen in Fig. 5(A), 14-3-3σ remained approximately constant over time in the

Fig. 4



NB1011 down-regulates cdc25C. (A) MCF7 cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for cdc25C expression (upper panel) and tubulin (lower panel). (B) MCF7TDX cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for cdc25C expression (upper panel) and tubulin (lower panel).

Fig. 5



NB1011 up-regulates 14-3-3σ. (A) MCF7 cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for 14-3-3σ expression (lower panel) and tubulin (upper panel). (B) MCF7TDX cells were treated with 30 μM NB1011 for 24, 48 or 72 h or left untreated. Cell lysates (30 μg) were examined for 14-3-3σ expression (lower panel) and tubulin (upper panel). Fold increase refers to a comparison of untreated and treated at each time point (relative to the level of tubulin) using a phosphorimager.

absence or presence of NB1011 in MCF7 cells. However, a significant increase in 14-3-3σ protein levels (as high as 5.9-fold) was observed in MCF7TDX following NB1011 treatment (Fig. 5B).

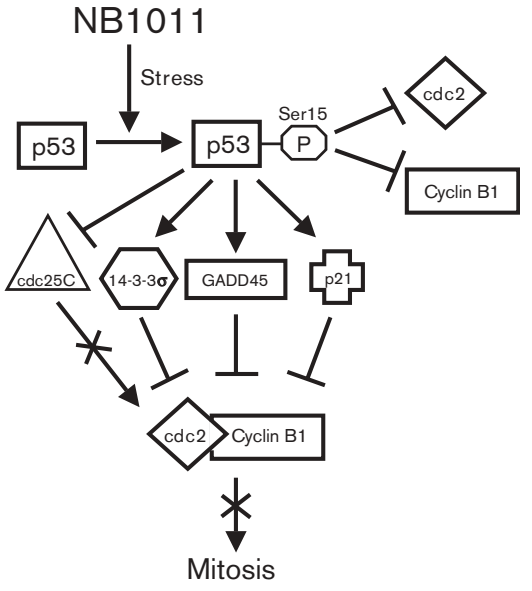
Discussion

NB1011 is a novel anti-cancer agent that specifically targets human tumor cells with elevated levels of TS *in vitro* and *in vivo* [11,12]. Overexpression of TS is a common mechanism of resistance employed by tumors against current chemotherapeutic drugs that function by inhibiting TS, such as 5-FU and TDX [4–6]. This makes NB1011 an ideal candidate for use in chemotherapy for patients who are not responding to classical TS inhibitors due to elevated intratumoral TS levels. Currently, NB1011 is being evaluated in phase I/II clinical trials in patients with colorectal cancer who have failed 5-FU based treatments. The detailed mechanism by which NB1011 exerts its cellular growth inhibition, however, remains to be elucidated fully. We have previously demonstrated that MCF7TDX tumor cells, possessing high TS levels, accumulate in the G_2/M phase of the cell cycle following treatment of NB1011 [12]. This accumulation correlated with an induction of p53, p21 and GADD45. In the present study, we examined the effect of NB1011 on the phosphorylation state of p53 and the expression of several key cell cycle regulators in high-TS-expressing tumor cells.

The tumor suppressor p53 becomes activated in response to stress signals. This activation is linked to stabilization of the p53 protein [13]. One major mechanism of p53 stabilization is phosphorylation of the N-terminus of p53, specifically Ser15, Ser20 and Ser37. Mounting evidence shows these phosphorylation events promote stabilization of p53 via two mechanisms, direct inhibition of the N-terminal nuclear export signal and by inhibition of MDM2 binding thereby preventing the ubiquitination and degradation of p53. Furthermore, phosphorylation of p53 enhances the direct binding of p53 to the promoter sequences of its target genes. Depending upon which target genes are affected, p53 induces a cell cycle arrest and/or apoptosis [13].

In a G_2/M arrest, cdc2, the kinase required for entry into mitosis, is inhibited simultaneously by various mechanisms. Three transcriptional targets of p53, p21, 14-3-3σ and GADD45, all play key roles in inhibiting cdc2 activity. p21 binds to and inhibits cdc2/cyclin B1 kinase activity directly, while 14-3-3σ sequesters the cdc2/cyclin B1 complex in the cytoplasm rendering it unable to initiate mitosis and GADD45 causes the dissociation of cdc2 from its regulatory subunit, cyclin B1, whose association is

Fig. 6



Schematic representation of the molecular mechanism for G_2/M checkpoint activation by NB1011.

required for kinase activity. Furthermore, p53 represses the transcription of cdc25C, cdc2 and cyclinB1, ensuring G_2/M checkpoint activation and subsequent cell cycle arrest [17].

In this report, we show that NB1011 treatment increases p53 levels and induces phosphorylation of Ser15 in tumor cells expressing high TS levels. This Ser15 phosphorylation is not observed in tumor cells that express low levels of TS and are not sensitive to NB1011. Following NB1011 treatment and subsequent p53 activation, the mRNA and protein levels of cdc2, cyclin B1 and cdc25C decreased, while the mRNA and protein levels of 14-3-3σ increased. Furthermore, the mRNA expression of p21 and GADD45 where confirmed to be up-regulated as well. Therefore, the cell growth-suppressive properties exhibited by NB1011 are due to direct inhibition of cdc2/cyclin B1 kinase activity through the classical G_2/M arrest paradigm as depicted in Fig. 6.

DNA damage is one of the main stress signals that results in the activation of p53 and the Ser/Thr kinase ATM has been identified as the primary kinase that is immediately activated upon the formation of DNA double-stranded breaks [21,22]. Activated ATM then phosphorylates many targets, including p53 on residue Ser15 [21–23]. Our results demonstrate that NB1011 treatment of high-TS-expressing tumor cells leads to the phosphorylation of p53 Ser15, implying the occurrence of DNA damage. Whether direct DNA damage is the cause of NB1011's cytotoxicity or whether it represents a secondary effect is still under investigation, since experiments performed with radiolabeled NB1011 did not show incorporation into DNA [33].

In summary, the data presented here show that NB1011 induces Ser 15 phosphorylation of p53 specifically in high-TS-expressing tumor cells. In addition, NB1011 activates the G_2/M checkpoint in MCF7TDX cells, while activation of this checkpoint is not observed in low-TS-expressing MCF7 cells, thereby exemplifying that NB1011 represents an excellent novel drug to treat patients with high intratumoral TS levels.

Acknowledgments

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