Abstract: NO donating iron nitrosyl complex with 2-aminothiophenyl ligand (2-AmPh complex) was studied for its ability to cause cell death and affect nuclear factor kappa B (NF-κB) signaling. The complex inhibited viability of HeLa cells and induced cell death that was accompanied by loss of mitochondrial membrane potential and characteristic for apoptosis phosphatidylserine externalization. At IC50, 2-AmPh caused decrease in nuclear content of NF-κB p65 polypeptide and mRNA expression of NF-κB target genes encoding interleukin-8 and anti-apoptotic protein BIRC3. mRNA levels of interleukin-6 and anti-apoptotic protein BIRC2 encoding genes were not affected. Our data demonstrate that NO donating iron nitrosyl complex 2-AmPh can inhibit tumor cell viability and induce apoptosis that is preceded by impairment of NF-κB function and suppression of a subset of NF-κB target genes.

Keywords: iron nitrosyl complex; 2-aminothiophenyl; apoptosis; NF-κB; p65; IL6; IL8; BIRC2; BIRC3
heme-containing enzymes (e.g., soluble guanylate cyclase), generation of reactive nitrogen species, and post-translational modifications [24–29]. All proposed mechanisms can underlie both pro- and anti-apoptotic effects of NO, and the resulting effects on the cell proved to be dependent on both NO concentration (and/or generation rate) and the cell type [24,25,28,29].

A number of transcription factors have been shown to be responsible for the effects of NO on cell survival [27,30–32]. Among others, the NF-κB signaling pathway has emerged during the last few decades as a target for pharmacological modulation for cancer therapy, since constitutive activation of NF-κB proved to be a common feature of most major human cancers [33,34]. The cell survival promoted by NF-κB involves activation of anti-apoptotic genes, e.g., anti-apoptotic Bcl-2 family members, the inhibitors of apoptosis proteins (IAPs) family, tumor necrosis factor receptor associated factor (TRAF), and others, which in turn confers resistance of cells to apoptosis triggered via receptor- or mitochondria-mediated pathways [27,33,35]. Thus, the paradigm of NF-κB pathway targeting in cancer therapy implies the inhibition of anti-apoptotic activity of NF-κB [36].

Inhibition of NF-κB by NO can occur via several mechanisms. NO donors impair nuclear localization of NF-κB [37–41] through stabilization of the inhibitor protein IkBα [41–44] that is a consequence of suppression of the inhibitory κB kinase (IKK) [43,44]. On the other hand, NO donors inhibit DNA binding of NF-κB [45–48]. Though the observed DNA binding suppression can be related to the impairment of nuclear translocation under certain experimental conditions, NO donors were shown to cause S-nitrosylation of NF-κB that accounts for the loss of DNA binding [46]. S-nitrosylation can be responsible also for other effects exerted by NO on NF-κB [49,50]. Yet another NO-induced posttranslational modification, the tyrosine nitration, has been demonstrated to lead to cytoplasmatic retention of p65 subunit of NF-κB [51].

Inhibition of NF-κB functions by NO leads to suppression of its anti-apoptotic target genes, which in turn augments apoptotic response in cancer cells [27,31,32]. Thus, nitric oxide exhibits versatile effects on NF-κB, and both NO and NF-κB are considered as important modulators of cellular functions relevant to the cancer therapy. Recently, NO-donor NIC bearing thioamine ligand with the amino group in the ortho-position of the phenyl ring, the 2-AmPh complex, has been synthesized and found to donate NO in a pH dependent manner and to be toxic towards a number of cancer cell lines of different origin [19].

Here we present data demonstrating that 2-AmPh induces apoptotic cell death in HeLa cells that is preceded by inhibition of NF-κB function as evidenced by the decrease in the nuclear content of p65 subunit of NF-κB and inhibition of some NF-κB target genes.

2. Materials and Methods

2.1. Synthesis of 2-AmPh

2-AmPh complex (Figure 1) was synthesized according to the method designated “method 1” in [19]. In brief, an aqueous mixture containing 0.3 g of KOH and 0.62 g of 2-aminothiophenol in 20 mL was added to 20 mL of aqueous solution of 0.496 g of Na2S2O3·5H2O and 0.57 g of Na2[Fe2(S2O3)2(NO)4]·4H2O under an argon atmosphere. The complex was obtained as a precipitate that was collected by filtration. After desiccation, the complex was recrystallized from acetonitrile.

![Figure 1. Structure of 2-AmPh.](image-url)
2.2. Cell Culture

The experiments were carried out on HeLa cell culture, M subclone (purchased from the Russian Collection of Cell Cultures of Vertebrates, Institute of Cytology RAS, St. Petersburg, Russia). The cells were cultured at 37 °C in an atmosphere of 5% CO₂ in the Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin).

2.3. Cytotoxicity Studies

Cytotoxicity was studied using MTT assay. Cells were plated in 96-well plates (5 x 10³ cells per well) 24 h before experiments in the standard incubation medium. The final DMSO concentration in all samples was 0.1%. After 24 h of incubation in the presence of 2-AmPh, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at the concentration of 0.45 mg/mL for 4 h, then the medium was aspirated and MTT-formazan was dissolved in 100 µL of DMSO. The staining intensity was measured at 570 nm and background absorption was determined at 620 nm. MTT staining of cells treated with DMSO was taken as 100%. The IC50 values were calculated using the median effect analysis [52].

2.4. Flow Cytometry

Cells were seeded to 10 cm cell culture dishes (10⁶ cells per dish). 24 h after seeding, cells were exposed to 2-AmPh at the IC50 for 24 h. Control cells were exposed to 0.1% DMSO. After exposure to test compounds, cells were collected by trypsinization and washed thrice with PBS (pH 7.4).

To study the cell cycle profile, cells were fixed and permeabilized by a dropwise addition of 70% ethanol pre-chilled to −20 °C followed by holding at 4 °C for at least 12 h. Ethanol from the fixed samples was removed by triple washing with 1% BSA in PBS, then cells were resuspended in staining solution containing 0.1% Triton X-100, 0.01 mg/mL propidium iodide (PI) and 0.1 mg/mL Rnase A in PBS, and incubated at room temperature for 30 min [53].

To study the externalization of phosphatidylserine (PS), cells were resuspended in 100 µL of annexin staining solution containing 5 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, 1 µL Annexin V-FITC solution (Sigma-Aldrich, St. Louis, MO, USA), 1 µg/µL 7-Aminoactinomycin D (7-AAD) and incubated for 30 min at room temperature. The samples were subjected to flow cytometry on a Guava easyCyte System (Millipore, Billerica, MA, USA) with Guava® Cell Cycle Assay software (guavaSoft™ 3.1.1, Millipore). Fluorescence was detected with a 488 nm excitation laser and a 695 nm emission filter for propidium iodide or 7-AAD, and 525 nm emission filter for annexin V-FITC.

2.5. Evaluation of Mitochondrial Membrane Potential

Cells were plated in 96-well plate in the standard incubation medium (8 x 10⁴ cells per well). 24 h after plating, 2-AmPh was added into the incubation medium at IC50. DMSO concentration in all samples was 0.1%. After 24 h exposure, the cells were washed once with PBS (pH 7.4) and stained using Mitochondrial membrane potential kit (Sigma-Aldrich) according to the manufacturer’s protocol. For positive control of the mitochondrial membrane potential loss, cells were exposed to an uncoupler of oxidative phosphorylation carbonyl cyanide m-chlorophenylhydrazone (CCCP) [54] at the concentration of 10 µM for 30 min. Fluorescence intensity was measured using plate reader Spark 10M (Tecan, Männedorf, Switzerland) with excitation/emission filter pairs 490/525 nm and 540/590 nm for monomeric and aggregated form of JC-1 dye, respectively. The mitochondrial membrane potential was estimated from ratios of emission intensities at 590/525. The 590/525 ratio in DMSO treated cells was taken as 100%.
2.6. Preparation of Nuclear Extracts and Immunoblotting

Nuclear extracts were obtained according to the described method [55] with slight modifications. Cells were plated and treated as described above in the “Flow Cytometry” section. In some experiments NICs containing cysteamine and phenylthiyl ligands, CysAm and Ph complexes, respectively, were used at IC50 doses that are 3.5 µM for CysAm [56] and 25 µM for Ph [57]. After exposure to the studied compounds for 6 h, cells were washed with PBS and lysed on ice for 15 min in the buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation/washing with the same buffer, pelleted nuclei were resuspended in the buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail. After 45 min stirring at 4 °C, the nuclear extracts were separated from residual nuclei by centrifugation for 15 min at 13,000 × g. Protein content in lysates was measured by the bicinchoninic acid method [58].

The proteins of nuclear extracts were separated in 10% PAGE, transferred onto Hybond-C Extra membranes (Amersham Biosciences, Little Chalfont, UK) and then blocked in TBST buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) supplied with 5% BSA and 0.02% NaN₃. After blocking, the membranes were incubated with NF-κB p65 primary antibody (Thermo Scientific, Waltham, MA, USA) followed by incubation with HRP-conjugated anti-mouse secondary antibody (R&D Systems, Minneapolis, MN, USA). The chemiluminescence reaction was carried out using the mixture containing 0.68 µM p-coumaric acid, 100 mM Tris-HCl, pH 8.5, 1.25 mM luminol, and 0.01% H₂O₂. The membranes were exposed to X-ray film and then stripped and reprobed with actin primary antibody (Sigma-Aldrich) and HRP-conjugated anti-rabbit secondary antibody (R&D Systems) to ensure equal loading.

2.7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells plated in 6-well plates were treated with 2-AmPh at IC50 or DMSO at the concentration of 0.1% for 6 h. Total RNA was isolated and purified using a GeneJET™ RNA Purification Kit (Thermo Scientific) according to the manufacturer’s protocol. The reverse transcription of the purified RNA was performed using MMLV RT kit (Evrogen, Moscow, Russia) and oligo-T-primer (Evrogen) according to the manufacturer’s instructions. qRT-PCR was performed using qPCRmix-HS SYBR reaction mixture (Evrogen). The primers used for gene expression analysis were as follows: IL6 sense 5’-TCCTGCAGAAAAAGCAGAAAT-3’, reverse 5’-AGCTGCGCAGAATGAGATGAG-3’; IL8 sense 5’-ACCGGAAGGAACCATCTCAC-3’, reverse 5’-GGCAAAACTGCACCTTCAC-3’; BIRC2 sense 5’-AGCGGGCCGTATCTCCTC-3’, reverse 5’-CTTCAGGGTTTGAATCGCAGT-3’; BIRC3 sense 5’-GGCGAGCGGATTCCGAGA-3’, reverse 5’-AACTACCTCCGAGATTAGACT-3’; and β-actin (ACTB) sense 5’-ACCGGGAATCGTGCGGTAC-3’, reverse 5’-AGCAGCGTGGCCCATCTC-3’. As a reference for normalization of the expression results, ACTB gene was used. Relative gene expression levels were computed using the REST 2009 software (QIAGEN, Hilden, Germany) from the results of three independent experiments.

3. Results

3.1. 2-AmPh Induces Apoptosis in HeLa Cells

In MTT staining experiments, 2-AmPh was studied in comparison with 2-aminothiophenol that was used for the complex synthesis. Both compounds decreased the viability in HeLa cells, but the cytotoxicity of 2-AmPh was appr. 2.5-fold higher compared to 2-aminothiophenol (Figure 2A). The IC50 value for the complex calculated from data of three independent experiments amounted to 29.7 ± 0.9 µM (expressed as mean ± standard deviation). Thus, for the further experiments, the complex was used at the found IC50 dose of 29.7 µM.

After 24 h exposure, 2-AmPh caused cell death as it was demonstrated by an increase in SubG1 population of cells revealed by flow cytometry of PI stained cells (Figure 2B). The increase in SubG1
population takes place at the expense of G2/M with no apparent changes in G1 or S fractions of cells. These results suggest that 2-AmPh causes cell death after either impairment of G2/M checkpoint or cell cycle arrest at phases preceding G2/M phases of the cell cycle.

The cell death caused by 2-AmPh was further characterized. The mitochondrial membrane potential loss is a well-characterized sign of cell death [59]. 2-AmPh induces a decrease in the mitochondrial membrane potential as revealed by JC-10 staining (Figure 2C). The complex also caused an increase in the number of cells stained by FITC-conjugated annexin V as it can be seen from Figure 2D. Annexin V binds PS with high affinity, and its binding to cells that are PI-negative (the lower right quadrant) is a well-defined marker of apoptosis-specific externalization of PS [60]. Thus, at IC50 2-AmPh induces apoptotic cell death in HeLa cells.

As it can be seen from Figure 2, the cell death did not account entirely for the decrease in cell viability. At the IC50, appr. 15% of cells are detected in the SubG1 population (Figure 2B), and no accumulation of late apoptotic or necrotic cells (Figure 2D, the upper right quadrant) was observed compared to the vehicle treated cells. These results show that cells with degraded DNA (SubG1 population) still retained their plasma membrane integrity since PI-positive populations of cells (Figure 2D, upper quadrants) did not increase. On the other hand, cell viability decreases by 50% after exposure to a test compound at IC50 dose. This discrepancy can be explained by the fact that MTT staining of cells depends rather on the mitochondrial electron transport chain functionality than the number of cells with intact DNA. Thus, the 50% decrease in cell viability measured by MTT does not have necessarily to be accompanied by an equal increase in population of cells with degraded DNA.

Our data show that 2-AmPh induces apoptosis in HeLa cells. Earlier, we have found that NICs with cysteamine or phenylthiyl ligands disturbed NF-κB functions [56]. This finding was consistent with other works demonstrating that NO-donor compounds affect NF-κB signaling [39,45–48,61–64]. Thus, further we studied the influence exerted by 2-AmPh on NF-κB.

![Figure 2](https://example.com/image2.png)

**Figure 2.** Cytotoxic properties of 2-AmPh for HeLa cells. (A) The “dose-effect” curve of MTT staining of cells. (B) The cell cycle profiles after PI staining of DNA. (C) Decrease in the mitochondrial membrane potential, as revealed from aggregated/monomeric JC-10 staining. **p < 0.001. CCCP was used as a positive control for the mitochondrial membrane potential loss.** (D) Flow cytometry analysis of cells stained with Annexin V-FITC conjugate and 7-AAD.
3.2. Effects of 2-AmPh on NF-κB Functions

The cell response to apoptotic stimuli initiates very early, and the alterations of NF-κB activity after NO donor exposure can be observed within several hours, long before the period of apparent cytotoxic effects. For example, the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) decreased the expression of c-Rel subunit of NF-κB in nuclei of murine leukemia L-1210 cells within 2 h, while the decrease in cell viability was observed after 24 h exposure [38]. The pattern of gene expression in response to SNAP has been demonstrated to change drastically, with main events taking place between 4 and 12 h [65]. Thus, to study the effects of 2-AmPh on NF-κB functions, we used a 6 h exposure time point.

NF-κB transcription factor is a dimeric protein comprising of two subunits. The most common composition of NF-κB is a heterodimer of p50 and p65 (RelA) polypeptides. The latter contains transcription activation domain, thus conferring transactivation function to the whole dimer [66]. With use of p65 specific antibody we have found that 2-AmPh affected the nuclear content of p65. After exposure to 2-AmPh the nuclear p65 level decreased substantially compared to the vehicle control (Figure 3A). The effect of 2-AmPh was compared to that exerted by NICs containing cysteamine and phenylthiyl ligands (CysAm [56] and Ph [57] complexes, respectively). All complexes were used at corresponding IC50 doses. Ph decreased p65 nuclear content to the extent similar to that observed under 2-AmPh exposure, while CysAm had a weaker effect on nuclear p65. Ph and CysAm were shown earlier to cause cell death and affect nuclear levels of p50 subunit of NF-κB [56,57]. It is interesting that CysAm exerted weaker effect on nuclear p50 compared to Ph. Thus, at equitoxic doses, NICs containing the phenyl ring (Ph and 2-AmPh) have more pronounced effect on the nuclear content of NF-κB subunits.

Since p65 subunit is important for NF-κB transactivation function, to find out if the decrease in nuclear p65 level can evoke functional consequences, we studied the expression of several NF-κB target genes representing two different functional groups, cytokine encoding genes IL6 and IL8, and genes encoding IAPs family proteins, BIRC2 (cIAP1) and BIRC3 (cIAP2). Under experimental conditions used, one of two cytokine genes, as well as one of two IAPs family genes tested, IL8 and BIRC3, were found to be inhibited by 2-AmPh, whereas IL6 and BIRC2 expression levels were not affected (Figure 3B).

Thus, apoptosis induction by 2-AmPh is preceded by the decrease in nuclear levels of transactivation domain containing NF-κB subunit p65 and inhibition of a subset of NF-κB target genes. Our data suggest that NF-κB signaling pathway is involved into the cell response to 2-AmPh.

![Figure 3](image-url)  
Figure 3. Disturbance of NF-κB function by 2-AmPh. (A) Decrease in the nuclear levels of p65 subunit of NF-κB revealed by the immunoblotting with anti-p65 antibody of nuclear extracts of cells treated with the vehicle, 2-AmPh and NICs bearing cysteamine (CysAm) and phenylthiyl (Ph) ligands. (B) Expression of NF-κB target genes after 6 h exposure to 2-AmPh relative to the control (0.1% DMSO) levels, * p < 0.05.
4. Discussion

NF-κB signaling is well recognized as an important molecular crossway that contributes to various diseases, including cancer [67–73]. Chemical compounds that inhibit NF-κB, either directly or via its upstream regulators, cause cell death and suppress tumor growth [74–83] or sensitize cancer cells to radiation or chemotherapeutic agents [84–86]. Nitric oxide, either produced by endogenous sources under conditions of hyperthermia [87], shear stress [88] and exposure to cytotoxic chemicals [89] or generated by exogenous NO donors [39,45], inhibited NF-κB signaling and caused loss of cell viability.

Previously, NO-donor complex 2-AmPh has been shown to be toxic to ovarian carcinoma (SKOV3), large intestine cancer (LS174T), mammary gland carcinoma (MCF7), and non-small cell lung carcinoma (A549) cells with IC50 values ranging from 25 to 74 µM [19]. So far, the mechanism(s) underlying the toxicity of 2-AmPh towards cancer cells has not been studied. Earlier, we have found that NF-κB is one of regulatory factors that are affected by NICs in HeLa cells [56]. Thus, we studied the effects of 2-AmPh on HeLa cells viability and NF-κB function.

The complex inhibited viability of HeLa cells with IC50 corresponding to the IC50 range found earlier for other cell lines. The IC50 of 2-AmPh is similar to that for HeLa cells of the structurally similar Ph complex [57]. Based on the results of MTT assay for 2-AmPh and 2-aminothiophenol (Figure 2A), it can be suggested that the toxicity of 2-AmPh might be partially accounted for by the presence of 2-aminothiophenyl moieties. On the other hand, the mechanism of decomposition of the complex in the course of NO generation is not known, and the resulting products that could be formed during the decomposition are not defined. Thus, determination of contribution of NO and other decomposition products to the overall toxicity of the complex would require more comprehensive studies.

At the IC50 dose, 2-AmPh caused cell death that was defined as apoptotic by annexin V staining. The apoptotic response of HeLa cells to 2-AmPh appears to be not completed by 24 h since the plasma membrane integrity was retained, and relatively small part of the cell population exhibited DNA degradation and PS externalization (Figure 2B,D). The mitochondrial membrane potential was not changed within the first 12 h of exposure and decreased only by 24 h (Figure 2C). These data demonstrate that the irreversible stage of apoptosis characterized by the mitochondrial membrane potential loss in the course of the apoptosis [90] was initiated after 12 h, hence supporting the finding that the execution of the apoptosis program was still ongoing by 24 h.

Cell death caused by 2-AmPh is preceded by inhibition of NF-κB function. 2-AmPh exposure caused the decrease in p65 levels in the nucleus (Figure 3A). Earlier, we have found that NICs with cysteamine or phenylthiyl ligands affected nuclear content of p50 subunit of NF-κB [56]. Thus, both present and previous data demonstrate that NO-donor NICs affect functions of NF-κB through modulation of its nuclear content. While our data are not sufficient to make a conclusion regarding if the NF-κB cytoplasmic-nuclear shuttling is affected by NICs, it is possible that NICs can affect the nuclear import of NF-κB as it was shown in many studies of NO donors [37–44].

The impairment of nuclear expression of p65 subunit is accompanied by the inhibition of a subset of NF-κB target genes. Since both NO and NF-κB play different (and often opposite) roles in the functions of the cell, we studied representative genes belonging to two groups with very different functions: Unrelated to apoptosis cytokine genes IL6 and IL8, and closely related to apoptosis IAPs family genes BIRC2 and BIRC3. Like many other NF-κB target genes, IL6, IL8, BIRC2 and BIRC3 are related to tumor progression and treatment: cytokines are involved into regulation of tumor microenvironment [91–93], whereas IAPs contribute to tumor cell survival [94–98]. The observed 2-AmPh-induced suppression of IL8 gene expression demonstrates that NO delivery to tumor cells might be beneficial for interfering with tumor promoting microenvironment conditions, e.g., angiogenesis [91]. The inhibitory effect of 2-AmPh on BIRC3 gene is consistent with the data on apoptosis induction by 2-AmPh. It must be noted that NO affects multiple signaling mechanisms in the cell [24–27], and its pro-apoptotic activity is not based solely on NF-κB regulation. Transcription factors p53, YY1 and FOXP3 have been shown to be modulated by NO [27,57] and can be involved in
NO-induced apoptosis. The observed inhibition of IAPs family gene BIRC3 can lead to decrease in anti-apoptotic capacity of cells and thus contribute to the overall apoptotic response.

Other studied NF-κB target genes, IL6 and BIRC2, were found to be not affected by 2-AmPh under our experimental conditions. The observed variations in the response of genes controlled by NF-κB on the action of 2-AmPh can be related to either different mechanisms of their regulation (e.g., requirement of specific accessory factors) or different kinetics of their response. Determination of mechanisms underlying this variability would require more detailed research.

5. Conclusions

NF-κB, an important factor regulating versatile processes in both healthy and tumor tissues, is considered as a possible molecular target whose inhibition might be beneficial in cancer treatment [33]. We demonstrate that NO-donor NIC with 2-aminothiophenyl ligand causes apoptotic cell death and suppress NF-κB target genes involved in cell survival and tumor progression.

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