Modulation of fenretinide induced cell death in neuroblastoma
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Favorable interactions between fenretinide and the Hsp90 inhibitor 17AAG in neuroblastoma

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Abstract

The efficacy and mechanism of action of 4HPR and 17 AAG were investigated in a panel of neuroblastoma cell lines and spheroids. In spheroids, the combination of 4HPR and 17AAG induced enhanced cytotoxicity compared to either of the drugs alone. A synergistic effect was observed in two out of four cell lines when cells were coinubated with both drugs. The synergistic effects of this combination might depend on the cells ability to suppress Hsp70-induction. Our results showed favorable interactions between 4HPR and 17AAG. Therefore, we feel that this combination might be a promising new strategy in neuroblastoma treatment.
Introduction
Fenretinide (N-(4-hydroxyphenyl) retinamide) (4HPR), a vitamin A analogue, induces apoptosis in various cancers, including neuroblastoma.\(^1,2\) 4HPR incubation results in induction of reactive oxygen species (ROS) and apoptosis in neuroblastoma.\(^1\) It is known that increased ROS levels may lead to the activation of the phosphoinositide 3-kinase (PI3K)/AKT survival pathway, which reflects a cellular response to the ROS-induced stress in the attempt to survive the drug insult.\(^3,4\) Heat shock protein 90 (Hsp90), a chaperone protein, is involved in maintaining the conformation, stability, activity and cellular localisation of several key oncogenic client proteins. Many of these client proteins are regulatory proteins or perform key functions in proliferation or modulation of apoptosis.\(^5-7\) Hsp90 activates the associated client protein to either bind ligand or be phosphorylated during signal transduction, as has been described for AKT.\(^8\) Inhibition of Hsp90 function, therefore, would abrogate the AKT survival pathway and sensitize cancer cells to certain anticancer agents that may otherwise activate the AKT protective mechanism.\(^9,10\) Several recent studies suggested that human cancer cells are very sensitive to inhibition of Hsp90, because Hsp90 is overexpressed in various tumour cells, including neuroblastoma.\(^11\) 17-allylamino-17-demethoxygeldanamycin (17AAG) exerts its antitumour effect by binding to the N-terminal ATPase domain of Hsp90 to inhibit its chaperone function.\(^9\) Preclinical tests have shown that 17AAG is non-toxic to normal cells, even though it is highly active against tumour cells.\(^10,12\) Cytostasis, cell-cycle arrest and apoptosis as a result of inhibition of RAS-RAF-pathway and PI3K/AKT-pathway were observed following 17AAG incubation in colon carcinoma and leukemia.\(^13,14\) Furthermore, 17AAG showed a potent synergistic toxicity when combined with other chemotherapeutics or irradiation.\(^14-22\) This highlights the potential use of 17AAG as single agent and as a sensitizer of chemotherapeutic drugs. By blocking ATP binding to Hsp90, activation of heat shock transcription factor (HSF1) might occur, followed by induction of Hsp70, which protects cancer cells from apoptosis (reviewed in Neckers CCR 2012\(^13\)).

In this study, we describe the beneficial effect of the combination of 4HPR and 17AAG in neuroblastoma monolayers and spheroids.

Materials and methods
Chemicals
4HPR (Sigma, St Louis, MO, USA), was dissolved in 100% ethanol, stored at 4° C and protected from light. 17AAG (Invivogen, San Diego, USA) was dissolved in DMSO at a 2.0 mM stock concentration and stored at -20° C. Serial dilutions were prepared
from the stock solutions with growth medium just before use. All other chemicals were purchased from Sigma (St Louis, MO, USA).

**Cell culture**

Three *MYCN* single copy neuroblastoma cell lines (FISK, NASS, SY5Y) and three *MYCN* amplified neuroblastoma cell lines (IMR32, SJ8, SJNB10) were cultured on RPMI-1640 culture medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum, 50 U/ml penicillin/streptomycin, 4 mM glutamine (Gibco, Invitrogen, Carlsbad, CA, USA) and 5 mg/l plasmocin (Invivogen, San Diego, USA). Cells were grown at 37° C, 5 % CO₂, in 95 % humidified air, all culture flasks and plates were from Corning (Corning, NY, USA). All cell lines were a generous gift of Prof. R. Versteeg (Dept. of Oncogenomics, Academic Medical Centre, Amsterdam). Spheroids were prepared from IMR32 and SJNB10, two cell lines well known for their capability of forming spheroids, as described before; 400,000 cells were grown in 4 ml culture medium in an ultra-low attachment sterile Petri dish. The spheroids were treated with a concentration range of 17 AAG (0-1000 nM) and/or 2 or 4 µM 4HPR for 2 weeks, without changing the culture medium. After treatment, the spheroids’ potency for regrowth was tested for 1 or 2 weeks by changing the medium with 17AAG for medium without 17AAG. The experiments were performed in quadruplicate. Growth curves were established by monitoring the spheroid size weekly by measurement of the cross-sectional area from five-times magnified photos using a microscope (microscope: Leica DMIL, Wetzlar, Germany. camera: SV camera, Lambert instruments, Leutingewolde, The Netherlands, Image Pro Plus 4.1).

**Measurement of cell viability**

Cells were plated in 96-wells plates and allowed to adhere overnight, after which the medium was replaced by medium containing different concentrations 4HPR (0-20 µM) and 17AAG (0-200 nM). After 72 h, the viability of the cells was measured using MTS assay according to the manufacturer’s protocol (Promega, Madison, WI, USA). The experiments were performed in quadruplicate. IC50-values for each drug were calculated from the dose-absorption curves.

**Immunohistochemistry**

Immunohistochemical analysis of sections of IMR32 spheroids was performed as described before. Apoptosis was detected using anti-cleaved caspase 3 1:100 (Bioke, Leiden, The Netherlands), proliferation was detected using anti-Ki-67 1:200 (Dako, M7240). Sections were examined using a ZEISS microscope and photographed using a Leica camera.
Expression of Hsp70

Hsp70 expression was measured in NASS and SJ8 cell lines. Both cell lines were cultured in 15 ml medium in a 75 cm² flask and allowed to adhere for 18 h, after which the medium was replaced by medium containing 4HPR (0-8 µM) and/or 17AAG (0-62.5 nM). After 24 h incubation cells were washed with PBS, then cells were lysed with RIPA buffer [1% (v/v) NP40, 5 g/l sodiumdeoxycholate and 0.1 % SDS in PBS) containing protease inhibitors (Roche) and phosphatase inhibitors (0.5 mM sodiumfluoride (Merck) and 0.5 mM sodiumorthovadate (Sigma)]. Cell lysates were kept on ice and protein concentrations were detected using a BCA protein assay (Thermo Scientific). Samples were mixed with sample buffer containing: 0.035 % (w/v) bromophenol blue, 17.5 % (v/v) glycerol, 10 % SDS, 0.2 M TRIS-HCl pH 6.7, 8.7 % (v/v) β mercaptoethanol and boiled for 5 min. Protein (15 µg) was loaded on a 7.5 % SDS/PAGE gel followed by Western blotting on nitrocellulose.

Western blotting was performed using the antibody: Hsp70 (Santa Cruz Biotechnology inc) 1:200 overnight and the secondary goat anti mouse antibody 1:2000 (conjugated with horseradish peroxidase) (Dako P0447). The antibody was dissolved in 3 % (w/v) BSA in 0.1 % (v/v) Tween 20 in PBS. After washing the membrane, detection of Hsp70 was performed with ECL plus (GE Healthcare Amersham). Equal loading was confirmed using a primary β-actin antibody in a 1:5000 dilution (Sigma) and a secondary goat anti mouse antibody in a 1:5000 dilution (Dako P0447).

Statistical analysis

Analysis regarding the efficacy of the combination treatment on growth inhibition was performed using the commercially available Calcusyn software (Calcusyn, Biosoft, Cambridge) designed by Chou and Talalay. The mode of interaction is reflected by the Combination Index (CI) indicating synergism CI < 0.9, additivity (CI 0.9 – 1.1) and antagonism (CI > 1.1). As CI values depend on the levels of growth inhibition (fraction affected (Fa)) data are presented in CI versus Fa graphs. Differences in spheroid growth were analyzed with the two sample t-test (SPSS 16.0).

Results

Viability after incubation with 4HPR and 17AAG.

In six neuroblastoma cell lines, the IC50 values for both 4HPR and 17AAG were determined after 72 h incubation. The IC50 values for 17AAG ranged from 51 nM to 151 nM (Table I). No correlation was observed between MYCN amplification and sensitivity to any of the drugs (Table I). IMR32, NASS, SJ8 and SJNB10 were co-incubated for 72
h with the 4HPR-17AAG-combination. The effect of the 4HPR-17AAG-combination was different in each cell line. Combination treatment in IMR32 and SJ8 cells showed an antagonistic effect, indicated by a CI > 1.1 (Fig 1). In NASS and SJNB10 cells, a synergistic effect of the combination was observed in cells treated with higher concentrations, indicated by a CI < 0.9. At lower concentrations, an additive effect was observed for the drug-combination in both cell lines (Fig 1).

Growth retardation in IMR32 and SJNB10 spheroids after incubation with 4HPR and 17AAG.

A strong concentration-dependent decrease in cross-sectional area was observed in IMR32 and SJNB10 spheroids treated with 17AAG for two weeks, with a complete growth arrest at highest doses (Fig 2A and 2B). Subsequently, medium of the spheroids treated with 1000 nM 17AAG was replaced by fresh medium to evaluate the cells’ potency for regrowth; in IMR32 spheroids, no regrowth was observed after 2 weeks of culturing in drug-free medium (Fig 2A). However, in SJNB10 a 1.6 x increase of the area was observed after 1 week of culturing in drug-free medium, indicating a slight increase in growth of the spheroids (Fig 2B). Additionally, IMR32 and SJNB10 spheroids were incubated with 2 or 4 µM 4HPR, respectively, and/or 500 nM 17AAG for 2 weeks. In both cell lines a significant decrease in area was observed in spheroids treated with the 4HPR-17AAG-combination compared with spheroids treated with single drugs (Fig 2C-D).

<table>
<thead>
<tr>
<th>Cellline</th>
<th>IC50 4HPR (µM)**</th>
<th>IC50 17AAG (nM)</th>
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<tbody>
<tr>
<td>FISK</td>
<td>11.7 ± 0.5</td>
<td>68 ± 34</td>
</tr>
<tr>
<td>NASS</td>
<td>10.1 ± 2.3</td>
<td>107 ± 29</td>
</tr>
<tr>
<td>SY5Y</td>
<td>2.7 ± 1.2</td>
<td>151 ± 31</td>
</tr>
<tr>
<td>IMR32*</td>
<td>1.0 ± 0.2</td>
<td>51 ± 10</td>
</tr>
<tr>
<td>SJ8*</td>
<td>4.1 ± 0.9</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>SJNB10*</td>
<td>2.9 ± 1.2</td>
<td>142 ± 15</td>
</tr>
</tbody>
</table>

Table 1. IC50 values of both 4HPR and 17AAG in six neuroblastoma cell lines. Six neuroblastoma cell lines were incubated for 72 h with a concentration range 4HPR or 17AAG; IC50-values were calculated from these data. *MYCN amplified cell lines ** Data from Cuperus et al.
Figure 1. Combination indices of neuroblastoma cells treated with the 4HPR-17AAG-combination for 72 h. A decline in CI was observed with higher fractions affected. Synergy of the 4HPR-17AAG-combination was observed in NASS (◊) and SJNB10 (♦) cells with a Fa > 0.75, indicated by a CI < 0.9. In IMR32 (■) and SJ8 (□) the combination effect was antagonistic indicated by CI values > 1.1. CI: Combination Index, Fa: Fraction affected.

Figure 2. The effect of 4HPR and/or 17AAG on spheroid growth. IMR32 and SJNB10 spheroids were treated for 14 days with 0-1000 nM 17AAG. At day 0, day 7 and day 14 the area of the spheroids was measured. IMR32 and SJNB10 spheroids were treated with 0 (♦), 125 (■), 250 (▲), 500 (□) or 1000 nM (∆) 17AAG. The culture medium of spheroids treated with 1000 nM 17AAG was replaced after 14 days incubation by drug-free medium. A. IMR32 spheroids. B. SJNB10 spheroids. C. IMR32 spheroids treated 14 days with 2 µM 4HPR, 500 nM 17AAG or the combination of both drugs. Area is depicted as percentage of t=0. * p-value < 0.05, ** p-value < 0.001.
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Figure 3. Immunohistochemistry of spheroid sections treated with the 4HPR-17AAG-combination. IMR32 spheroids were treated for 1 weeks with 10 µM 4HPR, 1 µM 17AAG or the combination of both drugs. Histological sections were prepared and stained with anti-cleaved caspase 3, a apoptosis marker and Ki67, a marker for proliferation. For color fig see page 162.
Combined treatment with 4HPR and 17AAG decreased proliferation and increased apoptosis in IMR32 spheroids.

Histological sections of IMR32 spheroids treated with 4HPR, 17AAG and the combination of these agents were stained with a proliferation marker, anti-Ki67 and an apoptosis marker, anti-cleaved-caspase-3 (Fig 3). In untreated spheroids a classical organized pattern of an outer proliferative rim and an inner apoptotic core was observed. In the single drug (4HPR and 17AAG) treated spheroids the classical rim-core pattern disappeared and the cell-cell structure became loser. The presence of apoptotic cells was observed as well as a decreased staining with the proliferation marker Ki67. The 4HPR-17AAG-combination resulted in a loose cell-cell structure, some cells stained positive for apoptosis and hardly any cell stained positive for proliferation. Additionally, morphological examination showed the presence of fussy cells with disintegrated membranes and cell debris, indicating apoptosis and necrosis. The cytotoxic effect of the combination of 4HPR and 17AAG was mainly due to a decreased proliferation, disintegration of the membrane and an increase in cell debris.

The effect of 4HPR and 17AAG on the Hsp70 expression

As mentioned above, NASS cells show a synergistic effect of the combination of 4HPR and 17AAG. An increase of Hsp70 expression was observed in NASS cells after incubation for 24 h with 17AAG only (Fig 4), this upregulation of Hsp70 was not observed upon co-treatment with 4HPR or with 4HPR treatment only. In contrast, in SJ8 an increase of Hsp70 was observed after incubation with either 17AAG and/or 4HPR (Fig 4). In SJ8 cells the 4HPR-17AAG combination has an antagonistic effect. Incubation with 17AAG results in a clear upregulation of Hsp70 and co-incubation with 4HPR did not abolished the Hps70 upregulation.

Figure 4. Immunoblot analysis of Hsp70 after 4HPR and/or 17AAG incubation. NASS and SJ8 cells were incubated for 24 h with a concentration range 4HPR and 17AAG and the combination of both drugs.
Discussion

4HPR, a vitamin A analogue, induces apoptosis in neuroblastoma, the induction of ROS has been shown to be one of the underlying apoptosis-inducing mechanisms. Hsp90 is a molecular chaperone protein that is induced in response to cellular stress and stabilizes client proteins involved in proliferation and anti-apoptotic signalling. For instance, Hsp90 is involved in AKT activation after stress induction [5-7]. Since oxidative stress has been associated with 4HPR induced apoptosis, we investigated the effect of the combination of 4HPR and Hsp90 inhibitor 17AAG in neuroblastoma cell lines and spheroids.

All cell lines of our panel were sensitive to both 4HPR and 17AAG, the sensitivity to both drugs did not correlate with MYCN amplification. The sensitivity for 17AAG in our panel of neuroblastoma cell lines is in line with results obtained by others [17, 19, 27, 28] and the IC50-values were lower than the clinical achievable plasma levels of 10-21 12-39 µM, measured in pediatric patients with various tumours, including neuroblastoma. [29-31] The combination of both drugs appeared to be synergistic in two out of four cell lines (NASS and SJNB10). The CI decreased with the fraction affected, indicating that the synergistic effect of the combination increased with the concentrations of either single drug.

In spheroids, which resemble micro metastasis in the avascular state, both IMR32 and SJNB10 showed profound growth retardation in the presence of either 17AAG or 17AAG combined with 4HPR. An increase in growth retardation was observed when spheroids were treated with the 4HPR-17AAG-combination compared to either of the single drugs. The effect of the combination was stronger in SJNB10 spheroids compared to that observed in IMR32 spheroids, which is in line with the results obtained in the monolayers. In immunohistochemical sections an increase of apoptosis and a decrease of proliferation were observed after treatment with both drugs, this effect is enhanced in sections treated with the combination. This is in contrast with immunohistochemical data of prostate cancer spheroids which suggested that growth control of spheroids exposed to the combination of 17AAG and irradiation is not mediated through enhanced apoptosis, but rather by a block in proliferation. [16, 22]

The expression of Hsp70 is of particular interest since this molecular chaperone has a well-documented anti-apoptotic function and its increase may reduce 17AAG induced cytotoxicity. [32, 33] Hsp70 expression was strongly increased in a concentration dependent manner after treatment with 17AAG which is line with results obtained by others. [28, 34, 35] In clinical trials, an increase of Hsp70 levels in peripheral blood mononuclear cells have been observed after treatment with 17AAG. [30, 31, 36] These increased levels of Hsp70 indicated induction of the cellular stress response following Hsp90 inhibition. [30] Hsp70 is known to be rapidly induced after chemical or physical stress, including oxidative stress.
4HPR induced an increase of Hsp70 only in SJ8 cells which might reflect the 4HPR-induced oxidative stress in this cell line.\(^1\)

The fact that the 4HPR-17AAG-combination induced Hsp70 expression in SJ8 cells but less in NASS cells might explain the difference in efficacy of the 4HPR-17AAG-combination between SJ8 and NASS, with SJ8 cells being less sensitive for the 4HPR-17AAG-combination.\(^10\) However, a conceivable possibility might be that in SJ8 cells the induction of Hsp70 protected the cells against the cytotoxicity normally induced by 4HPR and 17AAG.

In this study, we presented our data on the combination of 4HPR and 17AAG in neuroblastoma. Both drugs showed to be cytotoxic in all cell lines tested, in two out of four cell lines the combination of the drugs has shown to be synergistic. A possible protection mechanism of the cells against the cytotoxicity of the drugs might be the induction of Hsp70.

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References


Synergistic effects of fenretinide and 17AAG in neuroblastoma


