Modulation of fenretinide induced cell death in neuroblastoma
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Pleiotropic effects of fenretinide in neuroblastoma cell lines and multicellular tumor spheroids

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Abstract

The efficacy and mechanism of action of fenretinide (4HPR), a vitamin A analogue, was investigated in a panel of six neuroblastoma cell lines and multicellular tumor spheroids. The latter are three dimensional cell aggregates and as such, a model for micrometastases. In all cell lines, the production of ROS increased with 163-680 % after 1 h of treatment with 4HPR. In addition, a decrease of the mitochondrial membrane potential of 30-75 % was observed after 4 h of incubation with 4HPR. A 6-12 fold difference was observed between the IC$_{50}$ values for cell proliferation and viability between the most sensitive (IMR32) and most resistant (NASS) cell line towards 4HPR. Flow cytometric analysis showed an increased amount of apoptotic bodies and no cell-cycle arrest. The antioxidant Trolox completely inhibited the accumulation of 4HPR-induced ROS and prevented the 4HPR-associated cytotoxicity. In all neuroblastoma spheroids, 4HPR induced a complete cytostasis at clinical relevant concentrations (3-10 µM). Immunohistochemical analysis of 4HPR-treated spheroids showed a decreased staining for proliferation marker Ki-67 and an increased staining for cleaved-PARP, a marker of apoptosis. Our results suggest that 4HPR might be a promising agent for the treatment of micrometastases and high risk neuroblastoma.
Introduction

Neuroblastoma is the most common solid extracranial tumor in children. The prognosis for children suffering from neuroblastoma is highly dependent on the age at diagnosis and the stage of the disease. Patients suffering from metastasized neuroblastoma with amplification of the MYCN oncogene, which is found in approximately 25% of primary, predominantly metastasized neuroblastomas, have a very poor prognosis. Current therapy is based on treatment with chemotherapeutic drugs, surgery, treatment with $^{131}$I-meta-iodobenzylguanidine ($^{131}$I-MIBG) and autologous stem cell transplantation. Despite these aggressive therapeutic strategies, the likelihood for survival for patients suffering from high risk neuroblastoma has not increased significantly over the last 15 years. Therefore, new and effective therapeutic strategies are being developed to improve prognosis in these patients.

Stage 4s neuroblastoma is characterized by spontaneous regression of the tumor by differentiation. Retinoids are essential regulators of cell growth, differentiation and cell death. All-trans retinoic acid (ATRA) and 13-cis retinoic acid (13-cis-RA) have been shown to induce differentiation and death in neuroblastoma cells. Although treatment with 13-cis-RA increased the event-free survival in children with advanced-stage neuroblastoma, its use has been restricted by dose-limiting side effects and increased resistance of retinoic acid differentiated cells to chemotherapeutic drugs. Fenretinide (N-(4-hydroxyphenyl) retinamide) (4HPR) is a synthetic retinoic acid derivate which induced apoptosis rather than differentiation in a variety of tumors.

The precise mechanism underlying the apoptosis-inducing properties of 4HPR is not yet fully understood. It has been suggested that 4HPR can induce apoptosis by both retinoic acid receptor (RAR)-dependent and reactive oxygen species (ROS)-dependent pathways. Mitochondrial membrane depolarization, which plays a key role in the process of apoptosis, has been observed in some tumor types treated with 4HPR. However, it has been reported that 4HPR did not alter the mitochondrial membrane potential ($\Delta \psi_m$) in neuroblastoma. It is known that solid tumors require adequate diffusion of nutrients for tumor growth. Spheroids are three dimensional aggregates of cancer cells that, due to their cellular organization, have been shown to resemble in vivo tumors with respect to growth rates and sensitivity towards chemotherapeutic drugs. Most importantly, they resemble micrometastases during the avascular phase of their development, which provides the opportunity to study phenomena such as the penetration and effectiveness of cytotoxic agents on spheroid size. So far, the effect of 4HPR has not been studied on a panel of neuroblastoma multicellular spheroids combined with immunohistochemical evaluation. In this report, we describe the effect of 4HPR on various biological parameters in a panel of MYCN-single copy and MYCN-amplified neuroblastoma cell line monolayers as well as spheroids.
Materials and Methods

Chemicals
4HPR (Sigma, St Louis, MO, USA), was dissolved in 100% ethanol, stored at 4°C and protected from light. Serial dilutions were prepared from the stock solution with growth medium just before use. H₂O₂ (Sigma, St Louis, MO, USA) was prepared as a 100 µM stock solution in double distilled H₂O immediately before use. Trolox (Sigma, St Louis, MO, USA) was prepared as a 0.5 M stock solution in 100% ethanol and kept at -20 °C for one month.

Cell culture
Three MYCN single copy neuroblastoma cell lines (FISK, NASS, SY5Y) and three MYCN amplified neuroblastoma cell lines (IMR32, SJ8, SJNB10) were cultured in RPMI-1640 culture medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum, 50 U/ml penicillin/streptomycin and 4 mM glutamine. 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 Human Genetics, Academical Medical Centre, Amsterdam).

Spheroids were prepared by growing 400,000 cells in 4 ml culture medium in an ultra-low attachment sterile Petri dish. Because the cells could not adhere to the bottom of the dish they cohered, forming small aggregates. After three days, small spheroids (0.3-0.5 mm) were formed and these were individually transferred to ultra-low attachment 24-wells plates. One well contained one spheroid in 1 ml culture medium. Spheroids were treated with different concentrations 4HPR for three weeks, without changing the culture medium. The experiments were performed in quadruplicate. Spheroid size was monitored weekly by measurement of the cross-sectional area of individual spheroids 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 and proliferation
Cells were plated in 96-wells plates at a density of 5,000 or 6,500 cells per cell line per well in a total volume of 100 µl and were allowed to adhere overnight, after which the medium was replaced by medium containing different concentrations 4HPR (0-40 µM). After 24, 48 and 72 h medium with 4HPR was changed for fresh medium and the viability of the cells was measured with MTS assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. MTS incubation lasted for 4 hours at 37°C.
The effect of the antioxidant trolox on viability was investigated by incubating the cells with 4HPR (5 and 10 µM) and/or trolox (500 µM) for 72 h followed by the MTS assay. Cell proliferation was measured using a 5-bromo-2’-deoxyuridine (BrdU) incorporation assay (Roche Applied Science, Penzberg, Germany), according to the manufacturer’s instructions. Cells were incubated for 2 h at 37°C with BrdU labeling solution, without refreshing the medium. The experiments were performed in quadruplicate. The dose-absorption curves were used to derive the IC$_{50}$ values.

**Cell cycle analysis**

Apoptosis was evaluated by flow cytometry of propidium iodide (PI) stained cells. Cells (500,000) were grown in 4 ml culture medium in a 25 cm$^2$ flask and treated with 4HPR (1-5 µM) for 24, 48 and 72 h. Both attached and floating cells were harvested and fixed in 70 % ethanol in PBS and stored at 4º C. For flow cytometric analysis samples were centrifuged at 1000 x g for 1 min. Cell pellets were resuspended in 200 µl PBS after which 200 µl RNase-PBS in a 4 mg/ml concentration was added. Cells were stained with 400 µl PI-saponin (0.05 mg PI/ml 0.02% (w/v) (saponin-PBS), vortexed thoroughly and incubated for 10 min at 37°C. The stained cell nuclei were analyzed by a FACS Calibur flowcytometer (Beckton-Dickinson) and the data were analyzed using WinMDI1 version 2.8 software.

**Measurement of Apoptosis - PARP analysis**

Cells (750,000) were cultured in 4 ml in a 25 cm$^2$ flask and allowed to adhere overnight, after which the medium was replaced by medium containing 4HPR (0-20 µM). After 24 h, both attached and floating cells were collected and centrifugated and the pellet was resuspended in PBS, sonicated (2x20 s, 8 W) and boiled (5 min). Protein concentrations were detected using a BCA protein determination assay. Samples were diluted to equal protein concentrations in sample buffer. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed as described by Laemmli$^{21}$ followed by Western blotting using the primary mouse anti-human poly (ADP-ribose) polymerase (PARP) monoclonal antibody 1:10,000 diluted (Biomol, UK) and rabbit anti-mouse secondary antibody in a 1:5,000 dilution in PBS containing 3 % BSA and 0.01% Tween 20 conjugated with horseradish peroxidase. After washing the membrane, detection of PARP was performed with enhanced chemiluminescence. Equal loading was confirmed using Ponceau S staining.$^{22}$ Neuroblastoma cells treated with the histone deacetylase inhibitor BL1521 were used as a positive control.$^{23}$
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**Immunohistochemistry**

Spheroids were immersion fixed in buffered formalin and embedded in paraffin. Dewaxed and rehydrated sections (4.5 µm thick) were stained with hematoxylin and eosin (H&E) for morphology. Endogenous peroxidases were blocked with 3% hydrogen peroxide. After antigen retrieval (microwaving, TRIS/EDTA) aspecific binding was prevented by blocking with 5% normal goat serum. Apoptosis was detected using anti-cleaved-PARP 1:200 (BD Biosciences, USA), proliferation was detected using anti-Ki-67 1:200 (Dako, M7240), both incubated 1 h at room temperature. Sections were blocked using post-antibody blocking (Immunologic) for 15 min at room temperature. Sections were incubated with biotinylated secondary poly-HPR anti-rabbit/mouse antibodies and avidin-biotin complex (Vectastain ABC kit). Immunoreactivity was visualized by incubation with 3,3’-diaminobenzidine (Sigma, St Louis, MO, USA), tissues were counterstained with hematoxylin QS (Vector laboratories, USA). Sections were dehydrated, cleared in HistoClear II and mounted in DPX mounting for histology (Fluka, St Louis, MO, USA). For negative controls primary antibodies were omitted. Sections were examined using a ZEISS microscope and photographed using a Leica camera.

**Reactive Oxygen Species (ROS)**

ROS production was detected using CM-H$_2$DCFDA (Invitrogen, Molecular Probes). Cells (100,000) were cultured in 1 ml medium in 12-well plates and allowed to adhere overnight. Subsequently, cells were treated with a concentration range of 4HPR (0-10 µM) for 1, 2, 3, 6 and 24 h. The ROS responsive dye CM-H$_2$DCFDA was prepared in DMSO in a 5 µM stock solution and kept on ice until use. Culture medium was replaced by 0.8 ml freshly prepared CM-H$_2$DCFDA-solution and incubated at 37° C for 15 min. Subsequently, fluorescence was measured in a microplate reader (BMG labtech, FLUOstar optima); excitation 485 nm, emission 520 nm. Cells were harvested by trypsinization and protein determination was performed using Bicinchoninic Acid (BCA) reagents (Sigma) according to the manufacturer’s protocol using Bovine Serum Albumin (BSA) as a standard. ROS production was also measured after incubating the cells for 4 h with 20 µM 4HPR and/or trolox (500 µM).

**Mitochondrial membrane potential ($\Delta\psi_m$)**

Cells (200,000) were cultured in 1 ml culture medium in 12 well plates and allowed to adhere overnight. Subsequently, medium was replaced by medium containing different concentrations of 4HPR (0-20 µM). After 4-h incubation with 4HPR, medium was replaced by medium containing 10 µg/ml JC-1 (Invitrogen, Molecular Probes). After 15 min incubation at room temperature, the cells were washed with 500 µl PBS and the
ratio of monomeric form and aggregates was measured in a microplate reader (BMG labtech, FLUOstar optima); excitation 485 nm, emission 520 nm and 600 nm. In addition, the mitochondrial membrane potential was measured using 4µM tetramethylrhodamine methyl ester (TMRM) (Invitrogen). Fluorescence was measured with excitation A550 nm and emission A600 nm.

**Statistics**
Differences in viability between MYCN amplified and MYCN single copy cell lines were analysed using the two sample t-test (SPSS 14.0.2).

**Results**

**Effect of 4HPR on viability, proliferation and apoptosis in neuroblastoma monolayers**
A time- and concentration-dependent loss of viability and proliferation was observed in all six cell lines. The IC<sub>50</sub> values of the six neuroblastoma cell lines towards 4HPR are shown in Table 1. A 6-12 fold difference was observed between the IC<sub>50</sub> values both for cell proliferation and viability between the most sensitive (IMR32) and most resistant (NASS) cell line towards 4HPR. No significant differences in sensitivity towards 4HPR were observed between MYCN amplified and MYCN single copy cell lines.

<table>
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<td>SJNB10*</td>
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<tr>
<td>mean</td>
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*Table 1. IC<sub>50</sub> concentrations (µM) of all cell lines for 4HPR, cells were treated 24, 48, 72 h
Figure 1. Analysis of apoptosis induced by 4HPR. FACS analysis of PI stained cells; M1 represents the cells accumulated in the sub-G0 phase. The bars represent the percentage of cells in sub G0. Note: The difference in y-scale for IMR32 in the right hand panel.
To investigate whether this decrease in viability and proliferation was associated with apoptosis, cell cycle studies with 4HPR treated cells were performed (Fig. 1). A substantial amount of cells in sub-G1 phase were detected after incubation with 4HPR, which indicates DNA condensation and thus apoptosis. No cell-cycle arrest was observed. In addition to cell cycle experiments, the presence or absence of cleaved PARP was studied. In all cell lines, 50% of PARP was cleaved at a 4HPR concentration lower or equal compared to the IC_{50} concentration derived from the viability and the proliferation assay (data not shown).

**Effect of 4HPR on neuroblastoma spheroids**

Spheroids grown from each cell line were treated three weeks with a concentration range of 4HPR (0-10 µM). The spheroid size was monitored weekly by photo-imaging.

![Figure 2](image)

Figure 2. The effect of 4HPR on spheroids. Spheroids were treated with 4HPR and photographed weekly to monitor the increase of the area. A shows IMR32 spheroids treated with 4HPR. B shows the calculated area of spheroids treated with different concentrations 4HPR for 21 days, depicted as percentage compared to the untreated control. Each bar represents the mean area ± SD of four experiments. IMR32 cells were treated with lower concentrations of 4HPR than the other cell lines.
Figure 3. Histological analysis of spheroids treated with 4HPR. IMR32 spheroids were either treated 1 week with 4HPR (10-20 µM) or untreated (control). Spheroids were paraffin fixed and sections were stained with: A) H&E for morphology. B) Immunostaining with proliferation marker Ki-67. C) Immunostaining with apoptosis marker cleaved PARP. All sections were examined and enlarged 100x and 400x. For color fig see page 159.
The cross-sectional area of untreated spheroids increased exponentially in time. In contrast, spheroids showed a complete cytostasis when treated with high 4HPR concentrations (Fig. 2). The sensitivity for 4HPR in spheroids is similar to the sensitivity observed in monolayer, with the exception of NASS, which was approximately 10 times more sensitive when treated as a spheroid. An unexpected finding was that in SJNB10 and SY5Y spheroids, treated with low concentrations 4HPR, an increased area was observed with respect to the untreated control spheroids (Fig. 2).

Microscopical examination of H&E stained sections of untreated IMR32 spheroids showed an organized pattern of an outer proliferative rim and an inner apoptotic core. In the rim of untreated spheroids rosettes were visible, which are histologically typical for neuroblastoma (Fig. 3A). Apoptosis was observed by the presence of both picnosis and fragmented nuclei. In 4HPR treated spheroids, a looser cell-cell structure and a decreased amount of cells were observed (Fig. 3A). Immunohistochemical sections of IMR32 spheroids were stained with an apoptotic marker, PARP, and a proliferation marker, Ki-67. Treatment with 4HPR (10-20 µM, 1 week) showed a dose-dependent decrease in proliferation (Fig. 3B) and a dose-dependent increase in apoptotic cells (Fig. 3C).

Analysis of ROS and the mitochondrial membrane potential ($\Delta \psi_m$).

In all six cell lines, a dose-dependent increase of ROS production was observed from 1 h

![Figure 4](image.png)

*Figure 4. ROS production by 4HPR. ROS production measured by CM-H$_2$DCFDA fluorescence in six cell lines treated with 4HPR, depicted as percentage compared to the untreated control.*
until 6 h of incubation with 4HPR (Fig. 4). The production of ROS increased with 482-680% after 1 h of treatment with 4HPR in IMR32, SJ8, SJNB10 and SY5Y. In contrast, FISK and NASS showed only a modest increase in ROS (maximum increase was 212% and 163%, respectively) after treatment with 4HPR. In the presence of the antioxidant trolox, no accumulation of 4HPR-induced ROS occurred and trolox prevented the onset of 4HPR-induced cytotoxicity in all cell lines, except FISK (Fig. 5). The fluorescent dye, JC-1, accumulates in mitochondria in the presence of a normal $\Delta \psi_m$ and forms reversible aggregates (Fig. 6A). In case of a decreased $\Delta \psi_m$, JC-1 aggregates dissociates into the monomeric JC-1 molecule (Fig. 6B). In all cell lines, a similar dose-dependent decrease of the $\Delta \psi_m$ was observed after a 4-h incubation with 4HPR (Fig. 6C), these results were confirmed by a second probe, TMRM (data not shown). This indicates a disturbance of the $\Delta \psi_m$ induced by 4HPR, which could result in increased ROS production. The decrease in $\Delta \psi_m$ does not correlate with the degree of ROS production.

![Graph](image)

**Figure 5.** The effect of trolox on ROS and viability loss induced by 4HPR. A) ROS production measured by CM- H$_2$DCFDA fluorescence in cells treated with 20 $\mu$M 4HPR and 500 $\mu$M trolox for 4 h, depicted as percentage of the untreated control. B) Viability is measured using MTS-assay in cells treated with 5 $\mu$M 4HPR (NASS and SJ8 were treated with 10 $\mu$M) and 100 $\mu$M trolox for 72 h, depicted as percentage of the untreated control.
Figure 6. Changes in the mitochondrial membrane potential. A) Representative picture of untreated NASS cells incubated with the fluorescent dye JC-1. B) Representative picture of 4HPR treated NASS cells incubated with the fluorescent dye JC-1. C) Changes of the mitochondrial membrane potential was studied using the fluorescent dye, JC-1, in cells incubated 4 h with different 4HPR concentrations. For color fig see page 160.
Discussion

The synthetic retinoid 4HPR has been shown to have a promising preventative effect in recurrence of breast cancer, because of its apoptosis-inducing effect.\(^\text{24}\) A phase I study performed in neuroblastoma showed that 4HPR toxicity was moderate when compared to other retinoids.\(^\text{25, 26}\) Until now, most investigations of 4HPR in neuroblastoma have been restricted to one or a few cell lines and only in monolayers. Due to the heterogeneity of neuroblastoma, we have investigated the effectiveness of 4HPR \textit{in vitro} in a panel of six neuroblastoma cell lines. No significant difference was observed in sensitivity towards 4HPR in \textit{MYCN} amplified and \textit{MYCN} single copy cell lines. This is in contrast with Reynolds \textit{et al}\(^\text{27}\) and Wei \textit{et al}\(^\text{28}\) who suggested a positive correlation between \textit{MYCN} amplification and sensitivity to 4HPR in neuroblastoma. The effective concentrations of 4HPR inducing cytotoxicity in all cell lines were comparable with achievable plasma concentrations \textit{in vivo}. In all cell lines treated with 4HPR, apoptosis was detected by accumulation of apoptotic bodies in the sub G1-phase by flow cytometry and by PARP-cleavage. However, no cell-cycle arrest was observed in 4HPR treated neuroblastoma cells. In ovarian cancer cells, 4HPR induced a G2 arrest which was ascribed to the formation of 4-oxo-4HPR from 4HPR.\(^\text{29}\) Therefore, the fact that no cell-cycle arrest was observed in neuroblastoma cells might indicate that 4HPR is not metabolized to 4-oxo-4HPR in neuroblastoma cells.

The production of ROS has been described in various tumor cell types following exposure to 4HPR.\(^\text{30-32}\) ROS production has been measured in neuroblastoma with 4HPR and peak production was observed after 6 h.\(^\text{17, 30, 33-35}\) We observed ROS production already after 1 h of 4HPR incubation, indicating that ROS production is an early event in response to 4HPR incubation. Levels of ROS production correlated with the sensitivity of 4HPR measured in the viability assays, with the exception of FISK, being sensitive to 4HPR while producing only low levels of ROS. Trolox was able to prevent the accumulation of ROS and the loss of viability due to 4HPR in all cell lines although the protection was suboptimal in FISK. Thus, in FISK another mechanism, apart from oxidative stress, might be responsible for the induction of apoptosis by 4HPR. Although, the exact mechanism of ROS induction of 4HPR is still unknown, oxidative stress appears to be one of the main apoptosis inducing mechanisms.

Since the ROS producing effect proved to be very fast it is conceivable that 4HPR interferes directly with the mitochondrial respiratory chain.\(^\text{32}\) Apoptosis may occur when the amount of ROS produced in the mitochondria cannot be handled by radical-scavenging antioxidants.\(^\text{32}\) In contrast, some studies suggested that ceramide and gangliosides were essential in the induction of ROS via 12-LOX.\(^\text{30, 36, 37}\) Previously, it was shown that 4HPR did not alter the $\Delta\psi_m$ in the neuroblastoma cell line SY5Y.\(^\text{17}\) However, in our
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study a profound decrease in the Δψₘ was observed in all six cell lines after a 4-h incubation with 4HPR. This decrease in the Δψₘ might lead to cytochrome c release, and other mitochondrial enzymes, which in turn will activate caspase 9 to initiate the downstream processes of apoptosis.

For the first time, the effect of 4HPR on multicellular tumor spheroids was studied in a panel of neuroblastoma cell lines combined with histological analysis of immunostained sections. In 4HPR-treated spheroids of all six cell lines growth retardation and even cytostasis at higher concentrations was observed. However, SJNB10 and SY5Y spheroids treated with low concentrations (2 µM) 4HPR showed an increase in cross-sectional area compared to the controls. As shown by Marengo et al, ROS can stimulate cell proliferation when present in very low doses. Thus, it is conceivable that a small increase in ROS produced by low concentrations of 4HPR might result in increased growth in these spheroids compared to controls. Our results indicated an altered cell-cell structure in 4HPR treated spheroids. This mechanism might also explain the increased area in spheroids treated with low concentrations 4HPR. Nutrients might penetrate more easily due to the open structure allowing cells in the core of the spheroid to proliferate as well. Histological sections stained with an apoptosis marker, anti-cleaved-PARP and a proliferation marker, anti-Ki67 showed a concentration-dependent increase of apoptosis was observed after 1 week of treatment with 4HPR as well as a decrease in proliferation. Thus, the observed cytostasis (Fig. 2) is a result of decreased proliferation and induction of apoptosis and probably not a cell cycle arrest. Our results are in contrast with studies by Myatt et al who did not observe significant changes in the proliferation pattern in Ewing sarcomas. It is known that 4HPR also has an anti-angiogenesis effect on neuroblastoma cells. In this way, 4HPR prevents further development of micrometastases. Taken together, the cytotoxic effects of 4HPR on avascular spheroids and the anti-angiogenesis effect, suggest that 4HPR might be an effective chemotherapeutic drug for the treatment of micrometastases of different sizes.

Our investigations have shown that 4HPR induced loss of the mitochondrial membrane potential and ROS accumulation in neuroblastoma cell lines, which is accompanied by a strong induction of apoptosis. The potency of 4HPR to induce cytotoxicity in neuroblastoma monolayers and spheroids suggest that 4HPR might be a promising new agent in treatment of neuroblastoma.

Acknowledgements

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References


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