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The novel atypical retinoid ST1926 is active in ATRA resistant neuroblastoma cells acting by a different mechanism

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ABSTRACT

E-3-(4′-Hydroxy-3′-adamantylbiphenyl-4-yl)acrylic acid (ST1926) is a novel orally available compound belonging to the class of synthetic atypical retinoids. These agents are attracting growing attention because of their unique mechanism of antitumor action that appears different from that of classical retinoic acid. This study aims at investigating the antitumor activity of ST1926 in neuroblastoma (NB) preclinical models. In vitro, ST1926 was more cytotoxic than both its prototype, CD437 and all-*trans*-retinoic acid (ATRA) and it was active in the SK-N-AS cell line, which is refractory to ATRA. We showed that unlike ATRA, ST1926 does not induce morphological differentiation in NB cells where it produces indirect DNA damage, cell cycle arrest in late S-G2 phases and p53-independent programmed cell death. DNA damage was not mediated by oxidative stress and was repaired by 24 h after drug removal. The SK-N-DZ cell line appeared the most sensitive to the proapoptotic activity of ST1926, probably because both the extrinsic and intrinsic pathways appear involved in the process. Studies with Z-VAD-FMK, suggested that ST1926 might also mediate caspase-independent apoptosis in NB cells. In vivo, orally administered ST1926, appeared to inhibit tumor growth of NB xenografts with tolerable toxicity. Overall, our results support the view that ST1926 might represent a good drug candidate in this pediatric tumor.

1. Introduction

NB is the most common extracranial solid tumor of childhood and accounts for 7–10% of all childhood cancers. Despite progress with multimodal therapies, high-risk NB is still incurable in approximately 70% of cases [1]. NB arises from the neural crest cell precursors of the sympathetic nervous system failing to complete differentiation. This failure in terminal differentiation is the rationale for retinoid-based management of this malignancy. The Vitamin A analogue retinoic acids are essential regulators of cell growth, differentiation and apoptosis, and induce the differentiation of NB cells into mature neural cells [2]. Recently, a phase III trial in high-risk NB patients, which included intensive chemotherapy, autologous bone marrow transplantation, and pulse 13-*cis*-retinoic acid (13-*cis*-RA) therapy, significantly improved event-free survival compared to intensive therapy alone [1]. However, more than 50% of patients subsequently develop recurrent NB. The overall poor outcome of disseminated disease demands the development of new agents against NB.

Adamantyl retinoids or retinoid-related molecules (RRMs), represent a novel class of retinoids whose mechanism of action, although only partially understood, appears different from that of classical cyto-differentiating retinoids such as

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13-*cis*-RA and ATRA [3–5]. Indeed, RRMs induce apoptosis in ATRA-resistant cells [6,7]. RRMs can activate certain retinoic acid receptors (RARs, preferentially RARγ) and exert RAR-independent growth-regulatory or apoptogenic activities [8–10]. CD437, the prototype of this novel class of retinoids, was originally designed as a RARγ-selective agonist. CD437 is endowed with strong apoptotic properties in many tumor types, including NB [11,12]. However, the low bioavailability and therapeutic index limit the clinical potential of CD437 [13], leading to other RRMs with an improved pharmacokinetic profile, such as ST1926, which is completing preclinical development preliminary to Phase I trials.

ST1926 is a very promising agent which appears more apoptotic than CD437 in acute-promyelocytic leukaemia cells [13]. In different tumor models, CD437 and ST1926 induced DNA damage, cell cycle arrest in G1 or G2 phases and apoptosis [14,15]. Multiple mechanisms including activation of the mitogen-activated protein (MAP) kinases p38 and Jun N-terminal kinase (JNK), release of cytochrome c in the cytosol and subsequent activation of the caspase proteolytic cascade, are implicated in the apoptotic process triggered by RRMs [16–18]. There is evidence that reactive oxygen species (ROS) contribute to the apoptogenic activity of CD437 in NB [11]. It is still unclear whether nuclear DNA or the mitochondrion represents the primary target of CD437 and congeners [14,19]. However, there is increasing evidence that the mitochondria might be the direct target of RRM apoptotic activity [6,13,20]. In fact, it may be that RRMs increase cytosolic calcium by inhibiting mitochondrial uptake through unidentified molecular determinant(s) [13].

This manuscript reports for the first time on the antitumor activity of ST1926 in preclinical models of NB. We investigated the cellular pathways involved in the cytotoxicity of ST1926 in NB cells and its antitumor efficacy in NB xenografts. In vitro, NB cells sensitivity to ST1926, CD437 and ATRA (Fig. 1) were compared in both retinoic acid sensitive and refractory models. Cell cycle data and apoptosis induction in NB cells exposed to ST1926, were evaluated. The sensitive and versatile alkaline Comet assay was used to shed light on the nature of DNA damage induced by the atypical retinoid in our model. Our data with the pan-caspase inhibitor, Z-VAD-FMK, allowed us to explore the relative contributions of growth suppression versus apoptosis to the reduction in cell number/survival induced by ST1926 in SK-N-DZ cell line. Overall, these findings and the observed tumor growth delay in a panel of NB xenografts where the agent was tolerable, support interest for ST1926 as a potential drug candidate in NB patients.

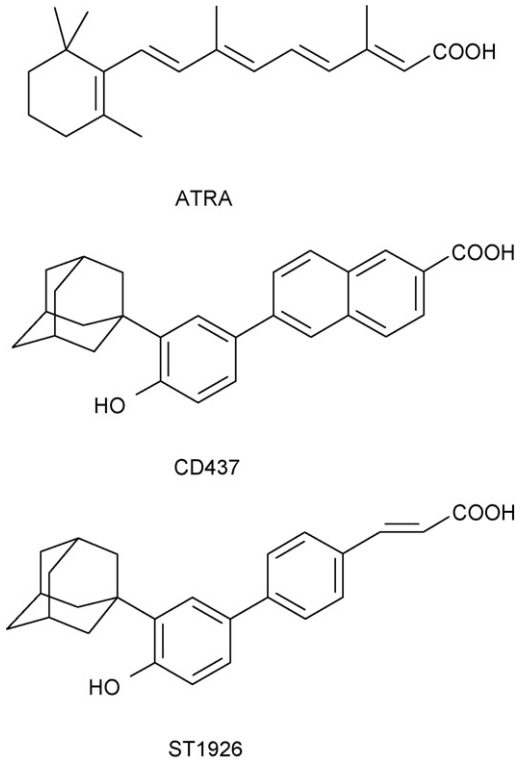


Fig. 1 – Molecular structures of ST1926, CD437 and ATRA.

2. Materials and methods

2.1. Cell culture and animal studies

A panel of five human NB cell lines with morphological and biochemically distinct phenotypes [SK-N-DZ, SK-N-BE(2)c, and SHSY-5Y: neuronal, N-type cells; SK-N-AS, SHEP-1: non-neuronal, S-type cells] from the ATCC, were used for the cytotoxicity studies. SK-N-AS cells were chosen as they are considered refractory to ATRA treatment [21]. The ovarian carcinoma cells IGROV-1 used as control in the Western blotting experiments, were kindly provided by Dr. Pisano (Sigma-Tau, Rome, Italy). Cells were maintained as monolayers in RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine under standard cell culture conditions (37 °C, 5% CO_2 in a humidified incubator). Five-week-old male nude CD1 nu/nu mice (Charles River, Calco, Italy) were used throughout the xenograft studies. All experimental animal investigations complied with the guidelines of the ''Istituto Superiore di Sanità'' (National Institute of Health, Rome, Italy) and the Ethical Committee of Catholic University, and all animal care was in accordance with local institutional guidelines.

2.2. Chemicals and reagents

ST1926 was kindly provided by Sigma-Tau, Pomezia, Rome. The drug was dissolved in DMSO to make the stock solutions (50 mM). CD437 was kindly provided by Dr. E. Garattini (Istituto di Ricerche Farmacologiche ''Mario Negri'', Milan, Italy). The stock solution was made in DMSO (10 mM). ATRA was purchased from Sigma and a stock solution of 10 mM was made in ethanol. All stock solutions were aliquoted, stored at −20 °C, protected from light and diluted to the final dilutions required for treatment in complete culture medium on day of experiment (the vehicle in the final drug suspension was less than 0.1%). Z-VAD-FMK, a general caspase inhibitor, was obtained from R&D Systems, BD Pharmigen. The following primary antibodies from Santa Cruz Biotechnology, USA, were used during our studies: mouse anti-p53 (DO-1), p21 (WAF1/Cip1) (187), Bcl-2 (SC509), β-actin, and rabbit anti-PARP (H-250), anti-caspase-8 p20 (H134) and anti-Bax (N-20)-G. Mouse

monoclonal antibody to human caspase 9 was purchased from Biodesign, USA.

2.3. Cell sensitivity studies

Cell sensitivity to the novel retinoid was assessed by either cell count with trypan blue (to assess viability upon drug treatment) and sulforhodamine B (SRB) assay (1, 6 and 24 h followed by recovery in drug-free medium up to a total of 72 h, and 72 h of continuous drug treatment) or a clonogenical survival assay (continuous treatment). In all assays, cells in exponential growth were seeded 24 h before incubation with the compounds. Cytotoxicity was assessed from dose–response curves as $IC_{30}/IC_{50}/IC_{70}$, which are the concentrations of retinoid required to inhibit 30/50/70% of cellular growth/colony formation, respectively.

2.4. Morphological studies

25×10^4 SHSY-5Y cells were seeded in duplicate into T25 flasks and incubated overnight in complete RPMI medium supplemented with 10% FCS. On the following day, ATRA (in ethanol) and ST1926 (in DMSO) were added at final concentrations of 5 μM and 0.05–0.1–0.5 μM, respectively. An equal volume of ethanol or DMSO was added to control plates. The culture medium was changed every 3 days and replaced with fresh medium containing the appropriate retinoid or control vehicle. At day 5, half the flasks were rinsed with PBS, and fresh drug-free medium was added. In the remaining flasks, the medium was replaced with fresh medium plus the retinoid, ATRA or vehicle and cells were cultured for another 4 days with fresh medium changes (containing the appropriate reagent) every second day. Cells were photographed after 2, 5, 7, 9 and 12 days.

2.5. Cell cycle perturbations

The human NB cell lines SK-N-DZ, SK-N-AS and SK-N-BE(2)c were selected from the initial panel used for cytotoxicity studies for further investigation. In the cell cycle analysis, about 1×10^6 cells were allowed to attach the day before, then they were either treated with cytotoxic concentrations of the compound (IC_{80}) at different times (24–48–72 h), or with increasing concentrations of ST1926 (IC_{30}, IC_{50} and IC_{70}) for 48 h. After each time point, cells were harvested and pooled with the original medium, washed in PBS and stained with 2 ml of propidium iodide staining solution (20 μg/ml propidium iodide in 0.1% sodium citrate), 25 μl RNAse (1 mg/ml in water) and 25 μl Nonidet P40 (0.15% in water) for about 2 h in the dark at room temperature. Cell cycle analysis was performed with a FACScan flow cytometer equipped with an argon laser (Becton Dickinson, Mountain View, CA). Twenty thousand cells were acquired and analyzed for DNA content with ModFit Version 2 software (BD).

2.6. Alkaline single cell gel electrophoresis (SCGE) or Comet assay

The Comet assay is a sensitive technique to detect DNA damage on a single cell level. It is a versatile method which allows the gathering of information on different types of DNA damage by simply modulating the pH of the buffer used for the unwinding and electrophoresis steps. At pH > 13 the Comet assay allows detection of a broad range of genotoxic damage comprehensive of DNA single (SSB) and double strand breaks (DSB), SSB associated with incomplete excision repair and alkali labile sites (ALS) which are expressed as SSB under the strong alkaline conditions of the assay. These conditions make the assay very sensitive and permit detection of SSB induced by an irradiation dose as low as 5cGy [22,23]. However, when the pH is above 12 and below 12.6 (to allow DNA denaturation and unwinding), the Comet assay detects all the described DNA damage except for the ALS sites [24,25]. Thus, the presence of increased migration at pH > 13, not observed at pH 12.1, is considered an indication of the specific induction of ALS. The assay was performed as previously described [26]. The following alkali unwinding buffers were used for 25 min at 4 °C: pH > 13: 300 mM NaOH, 1 mM EDTA; pH 12.1: 300 mM NaOH, 1 mM EDTA and concentrated HCl to adjust the pH. Electrophoresis was carried on at 23 V (0.7 V/cm), 300 mA for further 25 min at 4 °C. We further exploited the versatility of the Comet assay by adding to the standard procedure one more step involving proteinase K incubation. This modified version of the assay permits evaluation of whether the compound under study produces DNA–protein interactions. The increase in DNA migration upon proteinase K incubation, with respect to the classical Comet assay without proteinase K treatment, is an indication of the involvement of the proteins in DNA–drug binding. For proteinase K studies, slides were washed three times in TE buffer (10 mM Tris, 1 mM EDTA, pH 10), and then incubated in the presence of proteinase K (20 μg/ml) in the same buffer for 90 min at 37 °C. Among the different parameters used to quantify DNA damage, the percentage of DNA in the Comet tail (%Tail DNA) is considered the most useful as it bears a linear relationship to break frequency. We therefore expressed DNA damage by using this indicator.

2.7. Active caspase-3

The assay was performed according to the manufacturer's protocol (BD Pharmigen). Essentially, SK-N-DZ, SK-N-BE(2)c and SK-N-AS cells were allowed to attach overnight before incubation with equitoxic concentrations of the drugs (IC_{70}). After 48 and 72 h, cells were harvested and washed twice with PBS, then fixed with Cytofix/Cytoperm™ solution for 20 min on ice. Cells were then pelletted and washed twice with Perm/Wash™ solution before incubation with Phycoerythrin (PE)-conjugated monoclonal active caspase-3 antibody. After 30 min at room temperature in the dark, cells were washed with Perm/Wash™ and then analyzed by flow cytometer (FACScan, BD).

2.8. Western blotting

SK-N-DZ, SK-N-AS and SK-N-BE(2)c cells were treated in the presence of ST1926 (IC_{80}) or DMSO for increasing times (24-48–72 h). At the end of treatment, cells were washed in cold PBS, scraped on ice and lysed in ice-cold lysis buffer (0.5% sodium deoxycholate, 20 mM Tris–HCl, pH 7.4, 0.1 M NaCl, 1% Nonidet P40, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 5 μg/ml Aprotinin,

5 μg/ml Leupeptin, 50 mM NaF and 2 mM Sodium Orthovanadate) for 20 min on ice. The total protein extracts were quantified by the Bradford Biorad Protein Assay (Biorad) with BSA as standard. Equal amounts of protein were electrophoresed on acrylamide gels (10 or 12%) and the separated proteins transferred to a PVDF membrane. The membrane was incubated in 10 mM Tris–HCl (pH 7.2) containing 100 mM NaCl, 0.1% Tween 20 and 5% non-fat dry milk to block non-specific binding. The membrane was then incubated with the specific primary antibody, washed and treated with corresponding horseradish peroxidase-conjugated secondary antibody. After washing, antibody binding was visualized by chemiluminescent detection methods (Amersham, Biosciences, USA).

2.9. Studies with Z-VAD-FMK

The pan-caspase inhibitor Z-VAD-FMK was used to assay the role of apoptosis in the cellular response to ST1926 treatment in SK-N-DZ cells. $0.5–0.6 \times 10^4$ cells/well were seeded in a 6-well plate and left to attach overnight. On the following day, cells were treated with Z-VAD-FMK 10 μM (stock 20 mM in DMSO) for 90 min and then co-treated with the pan-caspase inhibitor and ST1926 at IC_{70} or IC_{80} for increasing times (24–48–72 h). The 10 μM dose of the pan-caspase inhibitor was selected because at higher concentrations (25 and 100 μM) the inhibitor proved to be toxic to SK-N-DZ cells. At the end of treatment, cells from each sample were counted with trypan blue to assess viability, and then processed for caspase-3 activation, cell cycle perturbations and Comet assay following the described procedures (see above).

2.10. In vivo growth inhibition analysis

We evaluated the effect of ST1926 on three NB cell lines (SK-N-DZ, SK-N-AS and SK-N-BE(2)c) growing as xenografts in nude mice. 30×10^6 cell were injected subcutaneously into nude mice in both flanks.

Two experimental schedules were utilized:

(a) *therapeutic study*: treatment was started after tumor was measurable.
(b) *preventive study*: treatment was started after tumor was palpable.

ST1926 was administered p.o. daily for 3 days over 3 weeks at various doses (15–20 and 25 mg/kg/day). The carrier vehicle was used as control. For the evaluation of antitumour activity, each control or drug-treated group consisted of 10–12 tumors. Tumor weight (TW) and total weigh were monitored every 3 days. TW was calculated by the formula: $TW = d^2 \times D/2$ (where d and D represent the shortest and the longest diameter, respectively). Antitumor activity was evaluated according to two criteria: (i) tumor weight inhibition (TWI) in treated (T) versus control (C) mice according to the formula: $100 - (T/C \times 100)$; and (ii) $\log_{10}$ cell kill (LCK) according to the formula: $(T - C)/3.32 \times DT$ (where T and C are the mean times [days] required for treated and controls tumors, respectively, to reach an established weight; DT is the mean doubling time of control tumors).

2.11. Statistical significance

Statistical significance of differences was determined by the Student's t test. The results were considered statistically significant if $p < 0.05$.

3. Results

3.1. ST1926 induces growth inhibition in NB cells

In order to establish the best treatment conditions for ST1926 in our model, the following incubation times were investigated in SK-N-DZ cells by a 5-day SRB assay: 1, 6, 24 and 72 h. The highest sensitivity of NB cells to ST1926 was observed following 24 h of drug treatment with the IC_{50} dose of 0.35 μM versus 3 μM and 5 μM obtained after 6 and 1 h, respectively. Seventy-two hours of continuous incubation did not appear to further enhance cell sensitivity to the compound. Therefore, we decided to use either 24 or 72 h of continuous treatment throughout the studies. The IC_{50}s from cell count experiments were between 0.13 and 0.23 μM (Table 1). Table 1 and Fig. 2A show that there was no significant difference in the ST1926 sensitivity between NB phenotypes. Furthermore, ST1926 produced a dose-dependent increase in nonviable cells that, after 72 h of incubation at doses of around IC_{70}–IC_{80}, reached or overcame the number of viable cells (data not shown). The IC_{50}s from the clonogenic assay were in the range of 0.035–0.050 μM with the SK-N-DZ cells being the most sensitive in the panel. These data indicate that about half the concentrations needed to produce cell number decrease (72-h treatment), were sufficient to inhibit colony formation. In SK-N-DZ cells, RRMs were between 30 and 100 times more effective than ATRA in producing cell growth inhibition, and ST1926 appeared significantly better than CD437 ($p < 0.05$) (Fig. 2B). Interestingly, when we compared ST1926 and ATRA sensitivity in SK-N-DZ and SK-N-AS cells with the SRB assay, we observed that whereas SK-N-AS cells were resistant to ATRA as previously reported [21], both cell lines showed similar

Table 1 – NB cells sensitivity to ST1926 following 72 h continuous exposure

μM	SK-N-DZ	SK-N-BE(2)c	SHSY-5Y	SK-N-AS	SHEP-1
IC_{30}	0.1 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.14 ± 0.02
IC_{50}	0.19 ± 0.04	0.15 ± 0.04	0.13 ± 0.01	0.13 ± 0.02	0.23 ± 0.02
IC_{70}	0.29 ± 0.06	0.22 ± 0.05	0.18 ± 0.02	0.18 ± 0.02	0.33 ± 0.02

Values are expressed as ±S.D. from three independent experiments.

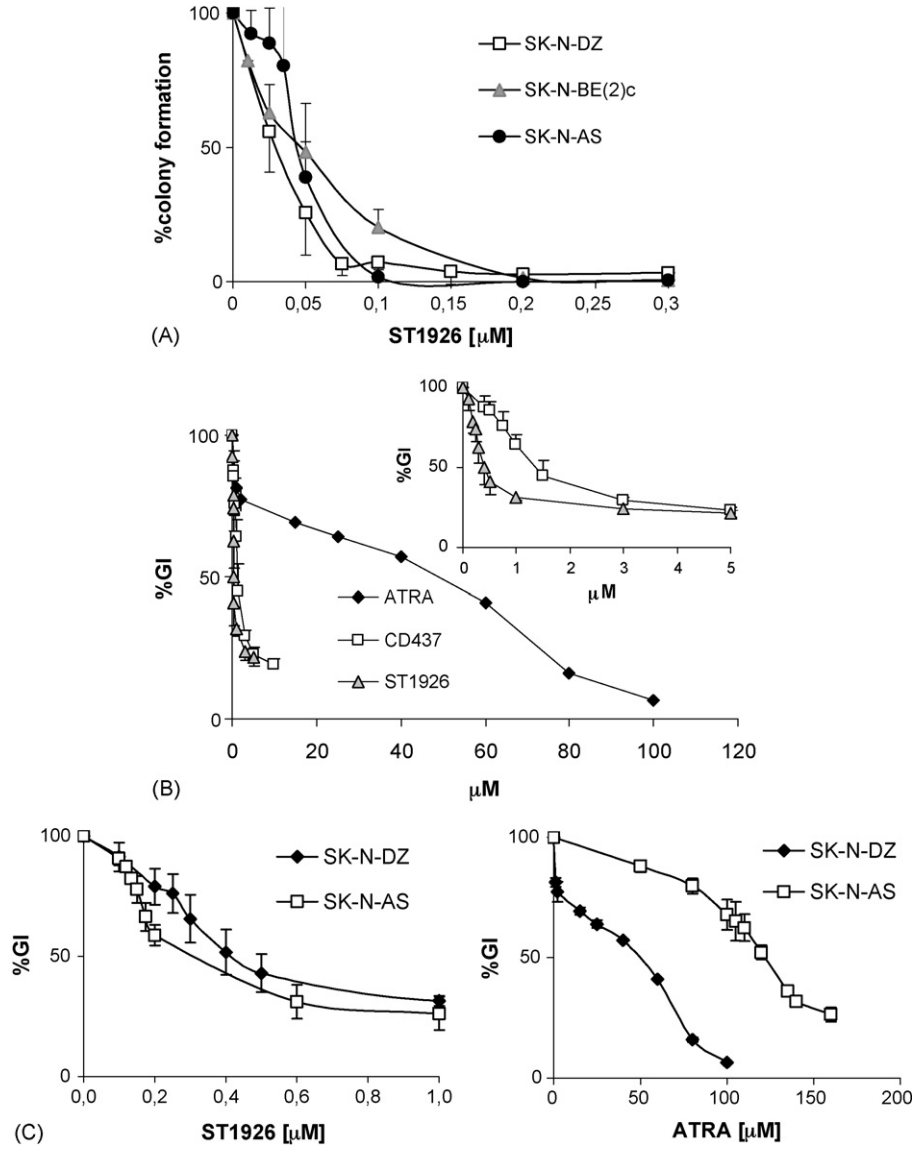


Fig. 2 – Cell sensitivity studies. (A) Clonogenic assay following continuous exposure of NB cells (SK-N-DZ, SK-N-BE(2)c and SK-N-AS) to increasing concentrations of ST1926. Values are expressed as ±S.D. from three independent experiments. (B) Five-day SRB assay following 24 h of incubation with the drugs (ATRA, CD437 and ST1926) in SK-N-DZ cells: growth inhibition (GI) curves. Insert: detail of %GI of CD437 and ST1926; $^{*}p < 0.05$ for 0.1–0.4–0.5 μM doses. Values are expressed as ±S.D. from three independent experiments. (C) Five-day SRB assay following 24 h of incubation with ST1926 and ATRA in SK-N-DZ and SK-N-AS cells. Values are expressed as ±S.D. from three independent experiments.

sensitivity to the atypical retinoid (Fig. 2C). Therefore, our data appear consistent with previous reports showing that atypical retinoids are active in ATRA-resistant NB cells [6,7].

3.2. *ST1926 does not induce morphological differentiation in NB cells*

Unlike ATRA, ST1926 does not appear to induce differentiation in SHSY-5Y cells (Fig. 3). Similar behaviour was observed in both the NB phenotypes (N- and S-type) used in the panel (data not shown). The only effect observed with ST1926 was a decrease in cell number upon treatment with the compound compared to untreated cells.

3.3. *ST1926 induces a late S-G2 arrest in NB cells*

To determine whether the ST1926-dependent reduction in cell number was associated with cell cycle perturbation, the distribution of cells in different phases of the cell cycle was measured at different incubation times (24, 48 and 72 h) with equitoxic dose (IC_{80}) of ST1926. In SK-N-DZ and SK-N-BE(2)c cells, ST1926 induced both S and G2 arrest that appeared earlier (24 h) in SK-N-DZ cells (Fig. 4A), and later (48 h) in SK-N-BE(2)c cells (Fig. 4B). On the other hand, in SK-N-AS cells the retinoid induced only S-arrest at all incubation times studied (Fig. 4C). A concomitant decrease in G0–G1 population was observed in all cell lines treated with equitoxic doses (IC_{80}) of

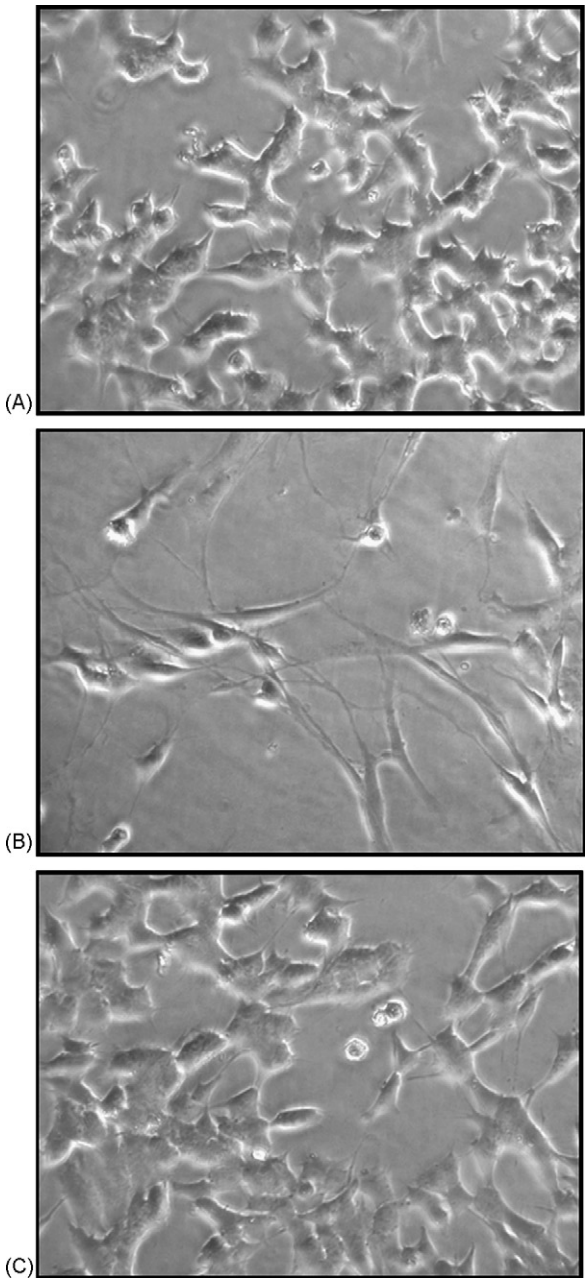


Fig. 3 – Morphological differentiation studies. SHSY-5Y cells treated with: (A) vehicle; (B) ATRA (5 μM); (C) ST1926 (0.1 μM) for 9 days according to the protocol described in Section 2. Neurite formation is evident only in ATRA-treated cells, whereas in ST1926-treated cells, there is a decrease in the number of cells compared to untreated control. Images from a representative experiment are shown.

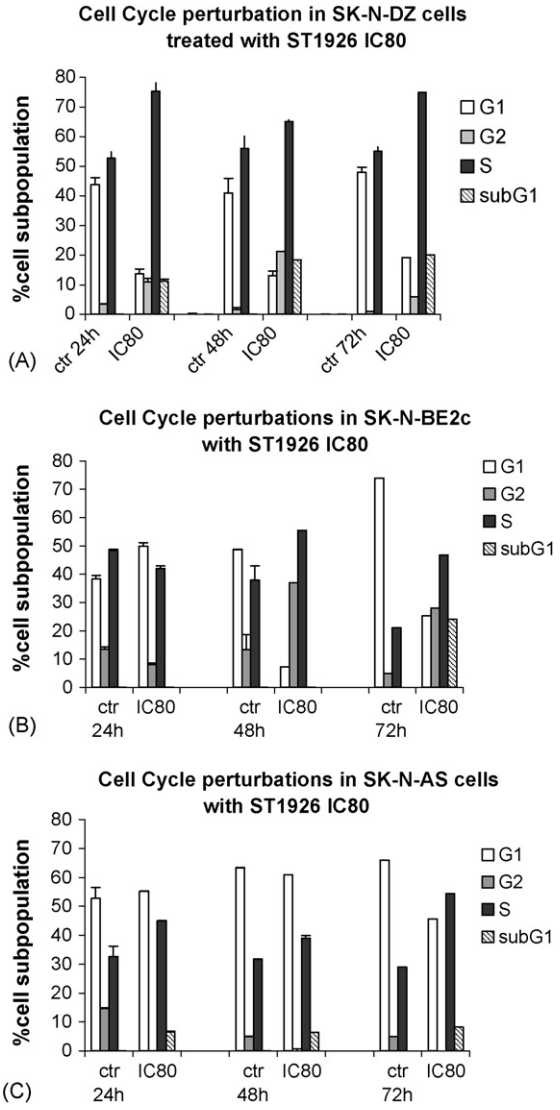


Fig. 4 – Cell cycle perturbations induced in NB cells: (A) SK-N-DZ; (B) SK-N-BE(2)c; (C) SK-N-AS) by ST1926 treatment (IC_{80}) for increasing times (24–48–72 h). Values are expressed as ±S.D. from three independent experiments.

the retinoid. Similarly, in the experiments run at a fixed time (48 h) and with increasing doses of ST1926 (IC_{30}–IC_{50} and IC_{70}), both SK-N-DZ and SK-N-BE(2)c cell lines showed an accumulation of cells in S phase at the lowest dose (IC_{30}) and in G2-M phase at the highest doses (IC_{50}–IC_{70}). On the other hand, in SK-N-AS cells, ST1926 appeared to induce mainly a dose-dependent S accumulation (data not shown).

3.4. *ST1926 is a genotoxic agent which interacts with DNA*

Although there are few lines of evidence that RRMs are able to induce DNA damage [14,15,19,27], it is still debatable whether DNA is the main target of the toxic action of these agents [3]. In order to investigate this aspect in our model, we run the alkaline Comet assay on NB cells treated with ST1926. Fig. 5A shows the Comet assay at pH > 13 on NB cells treated with increasing concentrations of ST1926 for 24 h and suggests that ST1926 is indeed a genotoxic agent. Rather than being limited to NB, this appeared a more general feature of this atypical retinoid because a similar finding was observed in the ovarian carcinoma cell line IGROV-1 and in leukaemia cell lines, where ST1926 and CD437 were compared showing both similar behaviour and DNA dama-

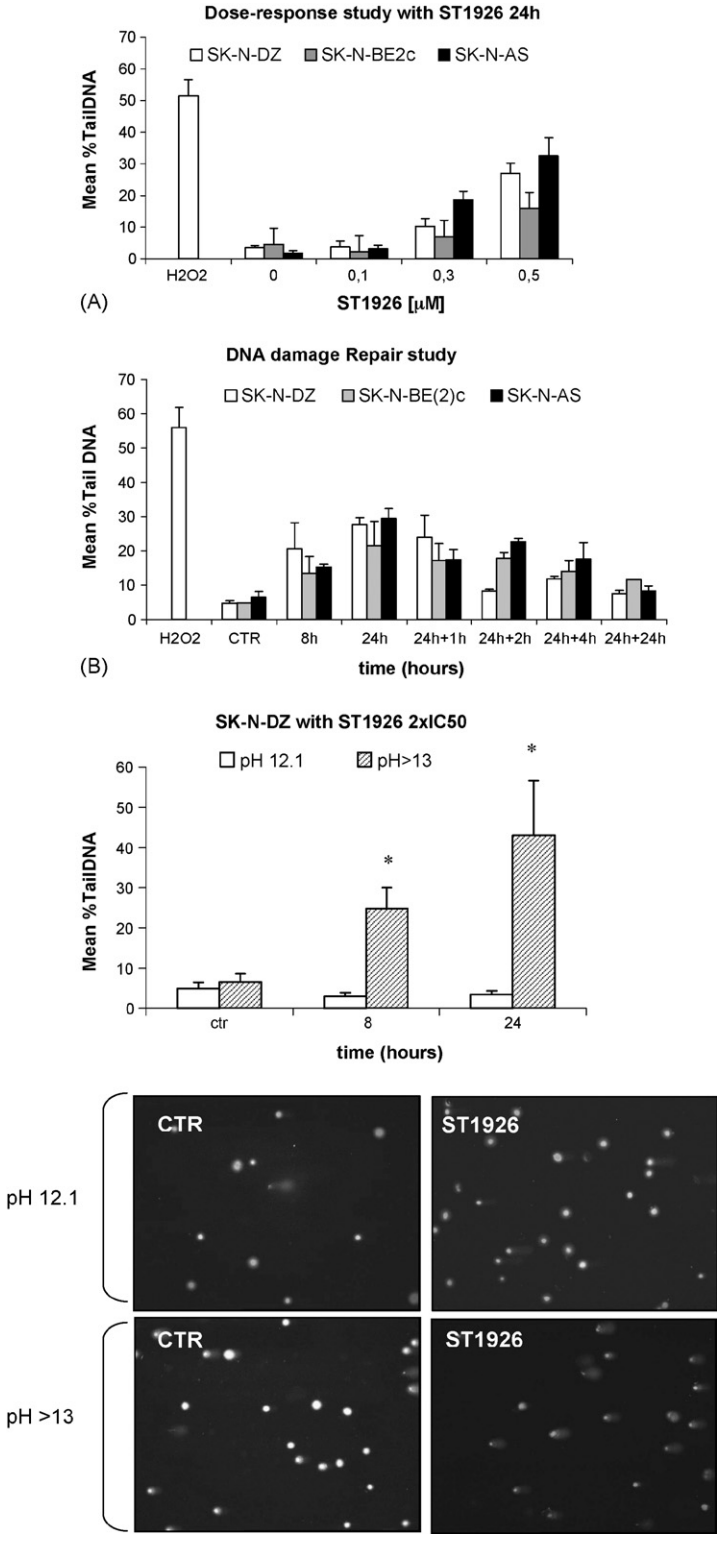


Fig. 5 – Alkaline Comet assay at pH > 13. (A) Dose–response curve following 24 h of incubation of SK-N-DZ, SK-N-BE(2)c and SK-N-AS cells with increasing doses of ST1926. On the X-axis, H_2O_2 indicates the internal control represented by SK-N-DZ cells treated with 50 μM H_2O_2 (5 min on ice). Values are expressed as ±S.E. from two independent experiments run in duplicate. (B) DNA damage repair studies in NB cells treated for either 8 or 24 h with ST1926 IC_{80}. After 24 h of incubation, cells were washed and left for increasing times up to 24 h in drug-free medium in order to evaluate the residual damage. H_2O_2-treated cells were included as internal control (see above). Values are expressed as ±S.E. from two independent experiments run in duplicate. (C) Comparison of Alkaline Comet assays at pH > 13 and pH = 12.1 on SK-N-DZ cells treated with ST1926 2× IC_{50} for 8 and 24 h. $^*p < 0.05$ compared with the corresponding samples run at pH 12.1. Values are expressed as ±S.D. from three independent experiments run in duplicate. Representative images from the study are shown below.

ging efficacy (data not shown). Among the NB cell lines studied, SK-N-BE(2)c cells appeared the most refractory to the toxic action of the retinoid on DNA. The initial damage, detected in all NB cells, appeared almost totally repaired after 24 h of cell growth in drug-free medium upon removal of the drug (Fig. 5B). More information about the nature of this damage comes from a the comparison between the Comet assays run at pH > 13 and pH 12.1. Fig. 5C shows no damage detected at the lower pH whereas at the higher pH we observed a significant damage already after 8 h of incubation at cytotoxic doses ($p < 0.05$). At pH 12.1, we had to stress the drug-incubation dose and time conditions up to 0.5 μM and 24 h, respectively, in order to see low but significant damage (>10% of Tail DNA) (data not shown). Furthermore, with the modified version of the Comet assay including proteinase K incubation (see Section 2), we did not observe any change in migration of damaged DNA with or without enzyme treatment, suggesting that ST1926 interacts with DNA but not with nuclear proteins (data not shown).

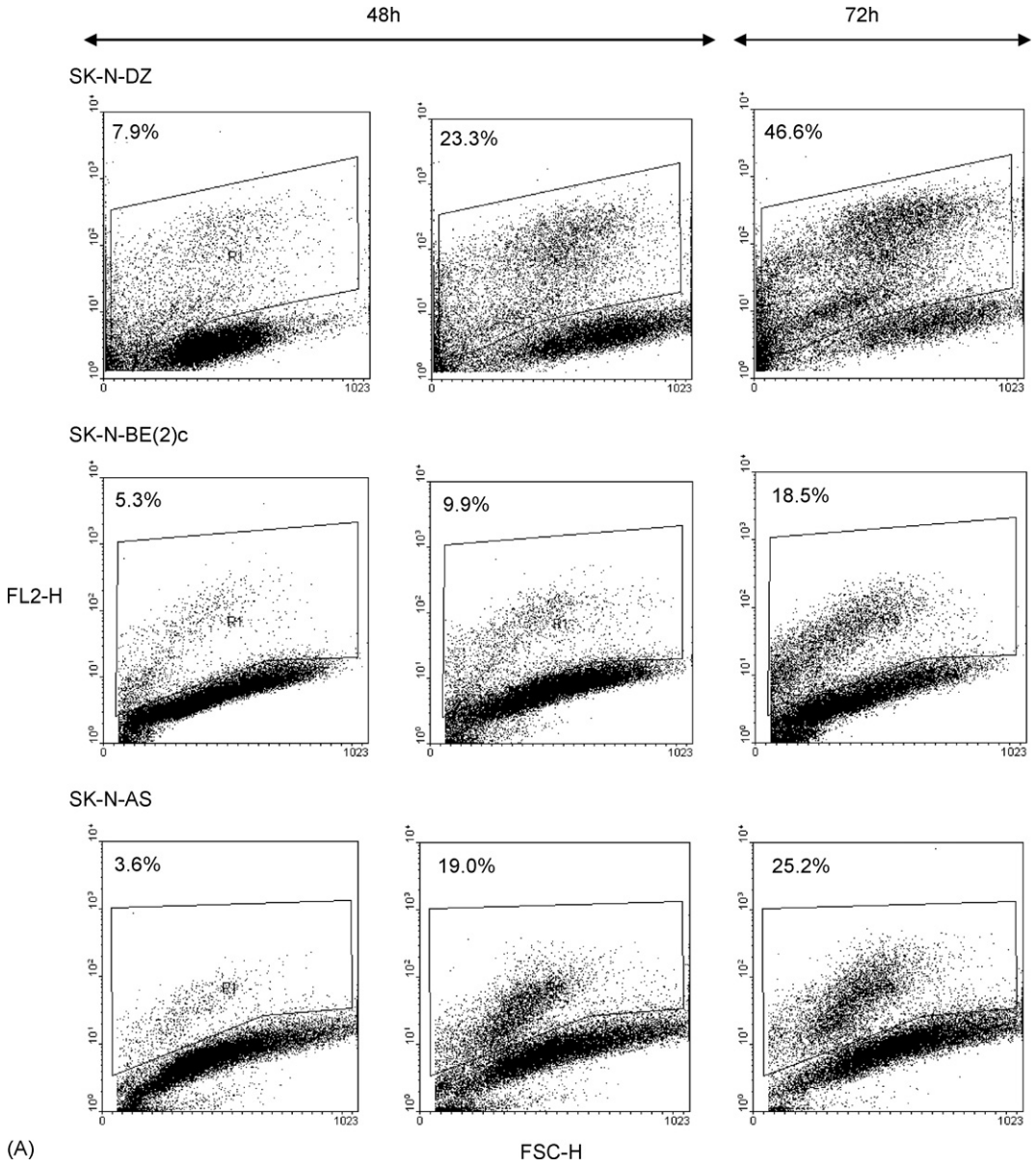


Fig. 6 – ST1926-induced apoptosis in SK-N-DZ, SK-N-BE(2)c and SK-N-AS cells. (A) Active caspase-3 experiment. Cells were continuously treated for 48 and 72 h with ST1926 IC_{70}, then incubated with PE-conjugated monoclonal active caspase-3 antibody. Dot plots of FSC-H vs. FL2-H (PE fluorescence), allowed to gate and quantify PE-positive events corresponding to active caspase-3 cells in drug-treated and control samples in all three NB cell lines. Dot plots from a representative experiment are shown. (B) Western blots on total lysates from NB cells treated with ST1926 IC_{80} for increasing times (6–72 h). The human ovarian carcinoma cell line, IGROV-1, treated with equitoxic dose of ST1926, was used as positive control for p21 and Bax expression. The activation of caspase-8 and caspase-9 was followed by monitoring the production of the p20 subunit and the 37 kDa active form, respectively. Images from one representative experiment are shown.

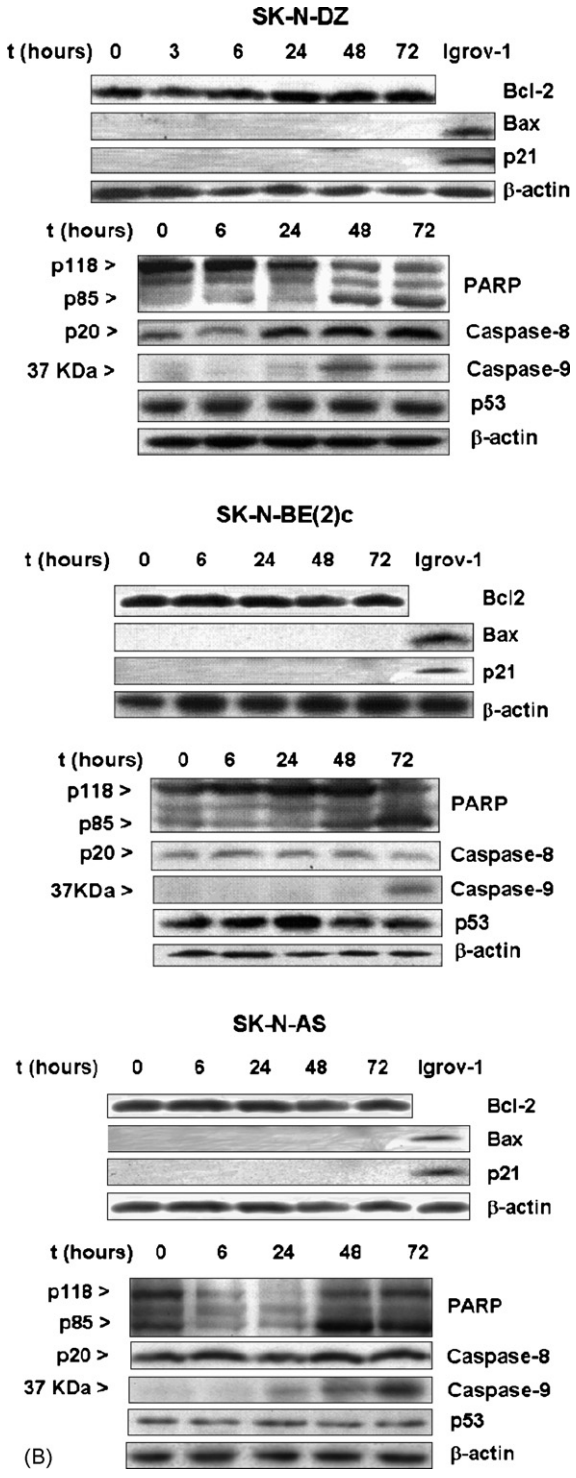
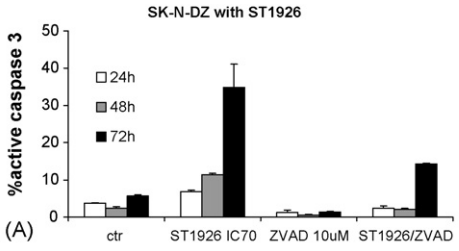
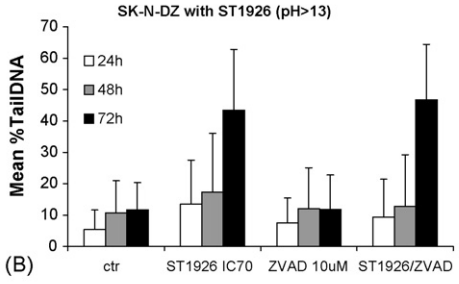


Fig. 6. (Continued).

3.5. *The novel retinoid induces caspase-dependent apoptosis in NB cells*

Atypical retinoids are considered strong pro-apoptotic agents in many different tumor types. To date, there is still little reported data about the mechanism(s) underlying RRM-induced programmed cell death in NB. CD437 induces caspase-dependent apoptosis of NB cells associated with free radical generation and mitochondrial-mediated apoptosis [28,29]. We found that ST1926-induced cytotoxicity was not mediated by ROS since pre- and co-treatment with Vitamin E did not affect either cell sensitivity to the retinoid or DNA damage induction by ST1926 (data not shown). SK-N-DZ and SK-N-AS cells were more sensitive than SK-N-BE(2)c cells to the both caspase-9 and -3 activation induced by the retinoid (Fig. 6A and B). Consistently, poly (ADP-ribose) polymerase (PARP) cleavage was clearly detectable following 48 h of ST1926 (IC_{80}) treatment in SK-N-DZ and SK-N-AS whereas in SK-N-BE(2)c it was observed not before 72 h of drug incubation (Fig. 6B). Interestingly, activation of caspase-8 was observed only in SK-N-DZ cells that appeared also the most sensitive to the activation of caspase-3 following ST1926 treatment with equitoxic doses (IC_{70}) (Fig. 6A). As SK-N-DZ and SK-N-AS carry wild-type p53, whereas SK-N-BE(2)c express a trascriptionally deficient p53 mutant gene [30], our findings would support the evidence that wild-type p53 tumor cell lines are more susceptible to RRM-induced apoptosis [31].





(C)

	CTR	ST1926 IC_{70}	Z-VAD-FMK 10μM	Z-VAD-FMK + ST1926
Viable cells ($x10^4$)	131.2±3.2 (100%)§	33.7±3.7 (26%)	135.9±8.7 (100%)	27.8±0.3 (21%)
Non viable cells ($x10^4$)	14.7±2.7	23.1±0.6	7.0±1.0	16.1±1.4
Sub G1	-	23%	-	6.1%
Cell cycle (%G1/G2/S)	45.6/6.1/48.3	36.6/16.3/47.1	43.5/5.14/51.3	30.3/26.5/43.3
DNA damage (Main %Tail DNA)	9%	27.5%	8.8%	36.3%

§Percentages of viable cells were calculated with respect to untreated control cells.

Fig. 7 – SK-N-DZ cells were treated with Z-VAD-FMK (10 μM) and ST1926 (IC_{70}) for increasing times (24, 48 and 72 h). (A) Effects of the pan caspase inhibitor on procaspase-3 cleavage induced by ST1926. Values are expressed as ±S.E. from duplicate samples. (B) Alkaline Comet assay (pH > 13) on the same samples from histogram A. Values are expressed as ±S.E. from duplicate samples. (C) SK-N-DZ cells were treated for 72 h with ST1926 IC_{80} in the presence or absence of 10 μM Z-VAD-FMK. Trypan blue, sub-G1, Mean %Tail DNA and cell cycle data are reported. Values are expressed as ±S.E. from two independent experiments run in duplicate.

3.6. Modulation of apoptosis-related proteins

To investigate the molecular pathways involved in the induction of apoptosis by ST1926 in NB cell lines, we evaluated the expression of apoptosis-related proteins over time (24–72 h) by Western blotting. We found that p53 was not modulated in any of the cell lines studied (SK-N-DZ, SK-N-AS and SK-N-BE(2)c). The Bcl-2/Bax ratio is known to influence cell death and survival. However, as Fig. 6B shows, either the level of these apoptosis regulators or their ratio were unchanged following ST1926 treatment in NB cells. Furthermore, p21 levels were not modulated by ST1926 (Fig. 6B).

3.7. Z-VAD-FMK studies

We wanted to shed some light on the relative contributions of growth suppression versus apoptosis to the reduction in cell number/survival induced by ST1926 in our model. If caspases play a major role in the toxic action of the retinoid, then the pan-caspase inhibitor, Z-VAD-FMK, would be expected to partially reverse the ST1926-dependent responses in our cells. For these studies, we selected the SK-N-DZ cell line, since it appeared the most susceptible to ST1926-mediated apoptosis. Treatment with 10 μM Z-VAD-FMK alone did not affect SK-N-DZ cell growth curves (data not shown). In drug-treated samples, Z-VAD-FMK did not affect ST1926-dependent reduction in the number of viable cells (Fig. 7C), whereas it reversed to control levels: the number of nonviable cells (Fig. 7C), the cleavage of procaspase-3 (Fig. 7A) and the accumulation of sub-G1 (Fig. 7C). In the presence of the pan caspase inhibitor, ST1926-induced G2 accumulation following 72 h of drug treatment appeared enhanced (Fig. 7C), whereas ST1926-mediated DNA damage was unaffected (Fig. 7B).

3.8. ST1926 is active in NB xenografts with tolerable toxicity

We evaluated the effect of ST1926 on three NB xenografts (SK-N-DZ, SK-N-AS and SK-N-BE(2)c), either by treating palpable tumors or by trying to prevent tumor development after cells inoculation. In both models, ST1926 was administrated p.o. for three consecutive days in a three consecutive weeks period a three dosage levels (15, 20 and 25 mg/kg/day); the carrier vehicle was used as a control. The results of the antitumor activity of ST1926 are reported in Table 2 and in Fig. 8. Significant tumor growth inhibition in a dose-dependent manner was demonstrated in the xenografts obtained from three cell lines using both the experimental designs. This effect was cytostatic rather than cytotoxic. In fact, the NB xenografts resumed a growth rate comparable with control within 1–2 weeks from the termination of treatment. In the prevention study, treatment with ST1926 resulted in better anti-tumor activity in terms of tumor growth inhibition and persistence of the effect: indeed, the LCK value has noticeably increased. Overall, ST1926 treatment was generally well tolerated by mice with no signs of acute or delayed toxicity. However, an approximately 10–15% reduction in body weight that was reversible upon cessation of treatment, was observed at the end of treatment period in mice receiving the highest ST1926 dose (data not shown).

Table 2 – Antitumor activity of ST1926 (p.o.) against human NB xenografts

Tumor	Dose (mg/kg)	Prevention study		Therapeutic study	
		TWI (%)	LCK	TWI (%)	LCK
SK-N-DZ	15	79	0.6	71	0.4
	20	78	1.7	84	0.7
	25	99	2.2	90	0.8
SK-N-BE(2)c	15	67	0.2	76	0.8
	20	83	0.5	78	0.8
	25	95	0.9	77	0.8
SK-N-AS	15	76	1.0	73	0.5
	20	81	1.1	84	0.7
	25	88	1.4	90	0.8

TWI: tumor weight inhibition; LCK: $\log_{10}$ cell kill.

4. Discussion

In this manuscript we presented lines of evidence that the novel retinoid ST1926 is active in a panel of NB cell lines and xenografts comprehensive of both the heterogeneity of this tumor type and a model not responsive to retinoic acid. We showed that ST1926 requires a minimum incubation time of 24 h to express maximum cell growth inhibition effects in NB cells, with not significant differences in sensitivity among the cell lines included in our panel. SK-N-DZ cells were more sensitive to RRMs with respect to ATRA and ST1926 was also significantly more effective than CD437 ($p < 0.05$) in this cell line. Similar results were observed in the SK-N-AS cell line which was refractory to ATRA, as already reported [21]. With the Comet studies, we showed that ST1926 is indeed a genotoxic agent whereas ATRA did not affect DNA (data not shown). A significant amount of damage (15–20% Mean Tail DNA) was detected at cytotoxic doses of the retinoid (IC_{80} from growth inhibition curves) already after height hours of incubation.

It should be pointed out that the role of genotoxic damage in the cytotoxic action of RRMs is still controversial [3]. To date, there are only a few lines of evidence that RRMs target DNA [14,15,19,27] and none of these reports used the sensitive alkaline Comet assay as detection method for DNA damage. Interestingly, our Comet data at pH > 13 suggest that at cytotoxic doses, ST1926 might specifically induce ALS due to some drug–DNA interactions without the involvement of nuclear proteins. This drug–DNA binding appears labile, as we showed that it was almost completely repaired within 24 h after drug removal. On the other hand, a significant direct production of DNA breaks was observed with the Comet assay at pH 12.1, only when we increased ST1926 doses and incubation times above the cytotoxic conditions. This direct DNA damage was still detectable in the presence of either Vitamin E or the pan-caspase inhibitor Z-VAD-FMK, suggesting that it is not mediated by ROS and it does not represent an early endonucleolitic DNA fragmentation associated with apoptosis. Overall, from the Comet data, we hypothesize that under cytotoxic conditions, the genotoxic action of the

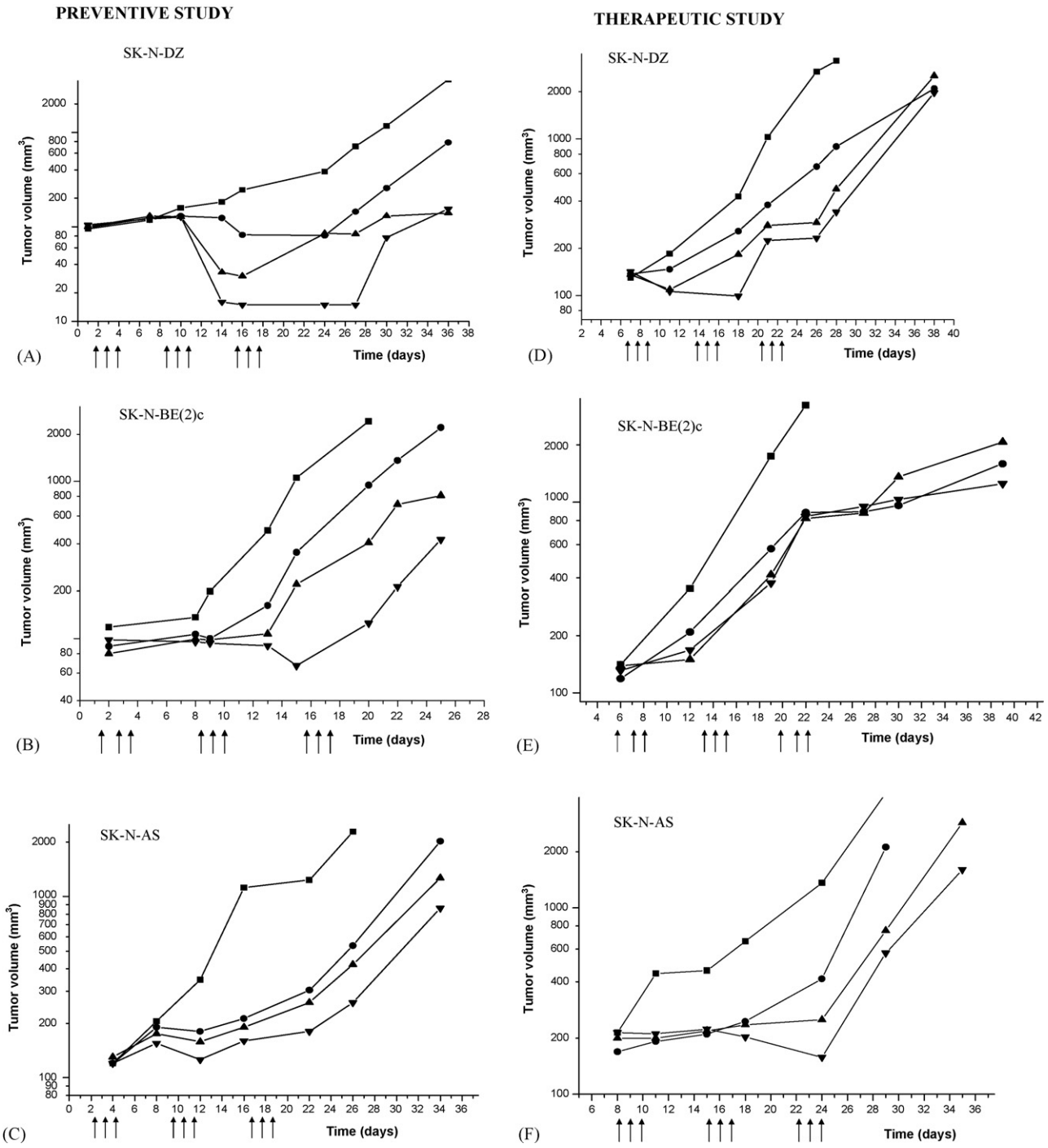


Fig. 8 – NB xenograft studies in CD1 nu/nu mice with ST1926 administered orally at increasing doses (15, 20 and 25 mg/kg/day) for three consecutive days and three consecutive weeks. (A–C) Preventive study: ST1926 was administered the day after tumor cells injection; (D–F) therapeutic study: ST1926 was administered when the tumor was palpable. Symbols: (■) control; (●) ST1926 15 mg/kg/day); (▲) ST1926 20 mg/kg/day); (▼) ST1926 25 mg/kg/day). Arrows indicate the days of treatment. Representative experiments are shown.

retinoid is likely an indirect effect due to drug–DNA interaction rather than the result of direct induction of DNA strand breaks. This hypothesis is consistent with a previous report by Zhao et al. showing that in S91 mouse melanoma cells, CD437 forms drug–DNA adducts, as detected by the nucleotide postlabeling technique [19].

All together, the data presented in this manuscript support the view that initially (8–24 h) ST1926 targets DNA in ways that can be detected as genotoxic damage by the Alkaline Comet assay (pH > 13). In response to DNA damage, cells start to accumulate in late S-G2/M phases in an attempt at repair, maintaining this block over time up to 72 h. DNA damage and

cell cycle arrest might play a major role in ST1926-induced decrease in cell number, as these effects persisted when caspase-3 activation was inhibited by Z-VAD-FMK treatment. Failure in DNA damage repair results in cells undergoing apoptosis (detected after 24–48 h) as suggested by caspases activation and PARP cleavage observed in all three NB cell lines. We argue that caspase-dependent apoptosis is one of the main modalities of cell death set in motion by ST1926 in NB cells, as the number of non-viable cells reversed back to the values of untreated control in the presence of the pan-caspase inhibitor. However, the inhibitor Z-VAD-FMK could not attenuate ST1926-mediated inhibition of colony formation in SK-N-DZ cells (data not shown), suggesting – as already described in other tumor models [32,33] – that caspase-dependent apoptosis is not the only contributor to ST1926-mediated cell death.

In our panel, SK-N-DZ cells appeared the most susceptible to retinoid-induced apoptosis. It is noteworthy that, only in this cell line, ST1926 appeared to activate both caspases 8 and 9. These data suggest that the involvement of both the extrinsic and intrinsic pathways of programmed cell death might play an important role in determining the final sensitivity of NB cells to the ST1926 pro-apoptogenic potential. Consistently with reports on other tumor models [13,32,34], we found that ST1926-mediated apoptosis in NB was p53-independent. We also showed that the expression of p53-regulated genes, p21, Bax and Bcl-2 was unaffected by ST1926 treatment. However, we observed that NB cells with a normal, active version of the protein (SK-N-DZ and SK-N-AS) were more susceptible than p53-mutated cells (SK-N-BE(2)c) to ST1926-mediated caspases activation. In fact, both pro-caspase-9 and the downstream substrate for caspase-3, PARP, were cleaved more rapidly (after 48 h) in SK-N-DZ and SK-N-AS cells with respect to SK-N-BE(2)c cells (after 72 h). This latter cell line was also the least susceptible to DNA damage induction by ST1926. Our data are therefore consistent with previous reports suggesting that the presence of wt-p53 might enhance responsiveness to RRMs [35,36].

One of the major objectives of this study was to evaluate the potential of ST1926 as antitumor agent in this pediatric tumor. NB xenograft studies were encouraging, showing that ST1926 was able to significantly slow tumor growth in a dose-dependent manner with tolerable toxicity. The atypical retinoid was administered orally for three consecutive days over a total period of three consecutive weeks, suggesting that the compound can better express its action over longer treatment times. In the prevention study, treatment with ST1926 resulted in a better anti-tumor activity in terms of tumor growth inhibition and persistence of the effect, as indicated by the increased LCK value.

It should be pointed out that in our *in vitro* studies, we did not observe any direct correlation between cytotoxicity on one side and ST1926-mediated DNA genotoxic damage and apoptosis on the other. In fact, growth inhibition curves indicated that all NB cell lines were similarly sensitive to ST1926 despite differences in DNA damage susceptibility and caspases activation. A detailed evaluation of the mode(s) of action of ST1926 in NB is beyond the objectives of this manuscript. However, although the data presented suggest that the mechanism(s) underlying ST1926 toxic activity in NB is(are) more complex, at the same time they add important information, supporting the idea that RRMs such as ST1926, represents a novel class of compounds with a different mechanism(s) of action with respect to retinoic acid. Indeed, we showed that unlike ATRA, ST1926 did not induce morphological differentiation in NB cells, producing only anti-proliferative effects. Furthermore, not only ST1926 was more active than both its precursor (CD437) and ATRA in NB cells, but it also induced cell growth inhibition, DNA damage and apoptosis in SK-N-AS cells that are refractory to retinoic acid. This is a very important observation since one of the limitations in the clinical use of retinoic acid in NB is represented by rising drug resistance.

Overall, we think that the data presented in this manuscript support the clinical potential of ST1926 in NB where the compound warrants further evaluation, either as a single agent or in combination.

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