Int. J. Cancer: **107,** 453–459 (2003)

Publication of the International Union Against Cancer

THE SYNTHETIC RETINOID ADAPALENE INHIBITS PROLIFERATION AND INDUCES APOPTOSIS IN COLORECTAL CANCER CELLS *IN VITRO*

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Chemotherapy of advanced stages of colorectal carcinoma is unsatisfactory. Retinoids inhibit cell growth and induce apoptosis in a variety of human malignancies. We compared the effect of the synthetic retinoid adapalene (ADA) and 9-*cis*-retinoic acid (CRA) on carcinoma cell lines *in vitro*. Colon carcinoma cell lines CC-531, HT-29 and LOVO as well as human foreskin fibroblasts were exposed to different concentrations of ADA and CRA for 3–72 hr. Proliferation was assessed by BrdU incorporation and apoptosis by FACS analysis. Breakdown of $\Delta\Psi_m$ was determined by JC-1 staining and activity of caspases 3 and 8, by a colorimetric assay. Quantitative Western blots were performed to detect changes in bax, bcl-2 and caspase-3. Both retinoic derivatives suppressed DNA synthesis and induced apoptosis in all tested cell lines time- and dose-dependently. While the natural retinoid CRA showed moderate antiproliferative and proapoptotic effects only at the highest concentration (10^{-4} M), the synthetic retinoic ADA was significantly more effective, showing remarkable effects even at 10^{-5} M. ADA and CRA disrupt $\Delta\Psi_m$ and induce caspase-3 activity in responsive tumor cells. Quantitative Western blots showed a shift of the bax:bcl-2 ratio toward proapoptotic bax in ADA-treated cells. Our results clearly indicate the superiority of ADA compared to CRA. Therefore, we suggest that ADA may be far more suitable as an adjunctive therapeutic agent for treatment of colon cancer *in vivo*.

Key words: *adapalene; synthetic retinoid; colon cancer; apoptosis*

Retinoids play a central role in cell growth and differentiation.[1] Physiologically occurring retinoids are ATRA and CRA (Fig. 1),[2] which effectively suppress growth of various tumor types, *e.g.*, skin, breast, lung, prostate, liver and pancreatic carcinomas.[3] ATRA and CRA act *via* binding and activation of specific nuclear receptors: RARs (RARα, -β, -γ) for ATRA and CRA action and RXRs (RXRα, -β, -γ), which are specific for CRA. These receptors belong to the nuclear steroid hormone receptor superfamily and act as ligand-dependent transcriptional regulators.[1] Advances in understanding RAR and RXR activation have led to a new generation of synthetic retinoids. ADA (CD271, 6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphtoic acid) is a naphtoic acid derivative with retinoid-like biologic properties and belongs to the third generation of retinoids (Fig. 1). It is commonly used in the topical therapy of acne vulgaris,[4] and its pharmacologic properties have been studied extensively.[5–7] ADA has been shown to inhibit proliferation of HeLa cells *in vitro* and to possess an anti-inflammatory potential[7] *via* inhibition of the lipoxygenase pathways.[1]

Retinoids inhibit colon cancer and hepatoma cell growth *in vivo*.[8,9] No data are available on the effect of the synthetic retinoid ADA on growth inhibition and apoptosis induction in different tumor types. We therefore investigated the effect of ADA on the colon cancer cell lines CC-531, HT-29 as well as LOVO and on human foreskin fibroblasts *in vitro*, comparing its proapoptotic and antiproliferative effects with CRA.

MATERIAL AND METHODS

Cell culture

CC-531 cells were cultured on 6-well tissue culture plates (Becton Dickinson, Mannheim, Germany) in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% FCS (GIBCO BRL, Karlsruhe, Germany), penicillin (107 U/l), streptomycin (10 mg/l) and ascorbic acid (50 mg/l) at 37°C and 5% CO_2. HT-29 and LOVO cells were cultured in the same medium without ascorbic acid. Human foreskin fibroblasts were maintained in DMEM (Biochrom) with the same additives (except ascorbic acid) and under the same conditions. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were starved for 24 hr in medium containing only 0.125% FCS, to keep most in the same stage of the cell cycle. Then, they were seeded at a density of 0.5×10^6/well and incubated for 3, 6, 12, 18, 24, 48 or 72 hr in the presence of ADA or CRA at 10^{-6}–10^{-3}M. ADA was dissolved in DMSO (Sigma, St. Louis, MO) and CRA in ethanol and then further diluted with full culture medium. After incubation, media were decanted, nonadherent cells saved and adherent cells trypsinized. All cells were centrifuged at 1,000*g* for 10 min and processed as described below. ADA was a gift of Galderma (Freiburg, Germany), and CRA was kindly provided by Hoffmann-LaRoche (Basel, Switzerland).

Flow-cytometric analysis of apoptosis

Cell death was measured by lysing cells in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide (Sigma) after washing twice with PBS and trypsin-EDTA solution. Labeled nuclei were analyzed on a FACSCalibur using CELLQuest software (both from Becton Dickinson). The percentage of apoptotic cells was determined by measuring the fraction of nuclei containing subdiploid DNA. Ten thousand events were collected for each sample analyzed.

Brdu-incorporation ELISA

DNA synthesis as a marker for cellular proliferation was measured by BrdU incorporation using the Cell Proliferation ELISA (Roche, Mannheim, Germany) based on incorporation of BrdU

Abbreviations: ADA, adapalene; AP-1, activator protein-1; ATRA, all-*trans*-retinoic acid; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; CRA, 9-*cis*-retinoic acid; CRABP, cellular retinoic acid–binding protein; CRBP, cellular retinol-binding protein; $\Delta\Psi_m$, mitochondrial membrane potential; DEVD, Asp-Glu-Val-Asp; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ERK, extracellular signal–regulated kinase; IETD, IIe-Glu-Thr-Asp; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NEM, N-ethylmaleimide; pNA, *p*-nitroanaline; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; RXR, retinoid X receptor; RXRE, retinoid X responsive element; TUNEL, TdT-mediated dUTP nick end labeling.

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Received 27 March 2002; Revised 18 July, 6 November 2002, 13 January 2003; Accepted 9 April 2003

DOI 10.1002/ijc.11410

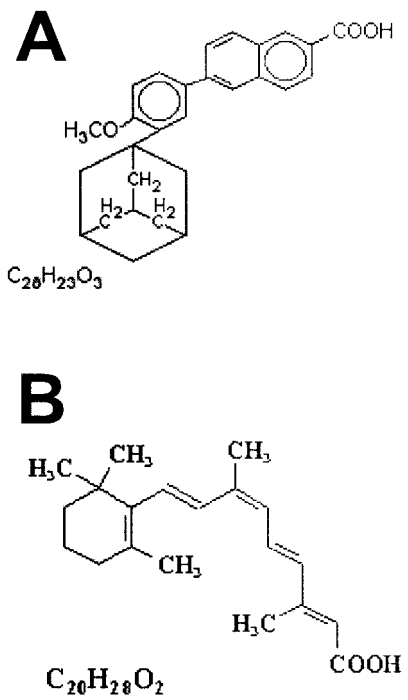

FIGURE 1 – Chemical structure of ADA (6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphtoic acid) *(a)* and CRA *(b)*.

into newly synthesized DNA and antibody-mediated detection of BrdU in DNA, as described before.[10] Briefly, cells were cultured as mentioned above but seeded at a density of 20,000/ml. BrdU was added together with the medicaments during the whole experimental period. Then, cells were fixed and DNA was denatured with an ethanolic solution provided by the manufacturer (30 min), followed by incubation with BrdU antibody (60 min, 37°C). Immune complexes were detected using tetramethylbenzidine for 5 min, the reaction was stopped with H_2SO_4 and absorption was measured at 450 nm in an ELISA reader (MRX II; Dynex, Frankfurt, Germany).

Analysis of $\Delta\Psi_M$

Mitochondrial injury was assessed by JC-1 staining (MoBiTec, Goettingen, Germany). This dye, existing as a monomer in solution emitting green fluorescence (537 nm), can assume a dimeric configuration emitting red fluorescence (597 nm) in a reaction driven by the $\Delta\Psi_m$.[11,12] Thus, red fluorescence of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to breakdown of $\Delta\Psi_m$.[13] Cells were adjusted to a density of 0.2×10^6/ml, trypsinized, washed in PBS, resuspended in 1 ml medium, stained with 5 μg/ml JC-1 for 15 min at 37°C and 5% CO_2 in the dark, then washed twice in PBS and resuspended in 0.5 ml PBS. Analysis was performed by FACS scan, and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria, detector FL-1) or red (intact mitochondria, detector FL-2) fluorescence.[11–15]

Immunofluorescence verification of apoptosis by M30 CytoDeath Antibody

Cleavage of cytokeratin-18 by activated caspases 3 and 7 reveals a neo-epitope that is specifically recognized by the M30 CytoDeath antibody (Roche). Generation of this neo-epitope is an early event in apoptosis, occurring before cells become positive for annexin V or TUNEL staining. This epitope is not present in nonapoptotic cells or tissues.[16,17] Cells were stained according to the manufacturer's instructions after 24 hr of incubation with 10^{-4} M ADA.

Assessment of caspase activity

The Caspase Colorimetric Assay (R&D Systems, Minneapolis, MN) was used to determine the enzymatic activity of caspases 3 and 8. The assay was performed according to the manufacturer's instructions after 24 hr of incubation with increasing concentrations of ADA. Caspase activation leads to cleavage of the provided colorimetric substrates (substrate peptides conjugated to pNA; caspase-3, DEVD-pNA; caspase-8, IETD-pNA) and can be measured photometrically at 405 nm. Raji cells treated with 10^{-5} M dexamethasone for 24 hr served as a positive control for caspase-8 activity.

Western blot analysis

Trypsinized and washed cells were lysed by adding 100 μl 2× sample buffer (2 mM NEM, 2 mM PMSF, 4% SDS, 4% DTT, 20% glycerol, 0.01% bromophenol blue, 2 M urea, 0.01 M Na-EDTA, 0.15 M TRIS-HCl) to 10^6 cells. DNA was sheared by pipetting up and down for 3 min at room temperature. Samples were boiled at 95°C for 15 min and centrifuged at 10,000*g* for 10 min. After separation by 14% SDS-PAGE (precast gels; Novex, San Diego, CA), proteins were transferred to nitrocellulose (pore size 0.2 μm, 240 mA, 30 min), which was blocked overnight at room temperature in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder, and nitrocellulose membranes were incubated for 90 min with either polyclonal rabbit antihuman Bcl-2 (1:400, sc-783), polyclonal rabbit antihuman Bax (1:500, sc-493; both Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal mouse antihuman caspase-3/CPP32 (1:1,000, C3172; Transduction Laboratories, Lexington, KY). Membranes were washed 3 times for 10 min in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder and incubated with an antirabbit IgG coupled to peroxidase (1:1,000, Sigma) for 1 hr at room temperature. Reactive bands were detected with the ECL reagent (Amersham, Freiburg, Germany).

RESULTS

ADA inhibits DNA synthesis more effectively than CRA

DNA synthesis as a correlate of cell proliferation, as determined by BrdU-incorporation ELISA, was set to 100% in untreated cells. At 10^{-5}M, ADA reduced DNA synthesis to 24.6% in CC-531, 56.0% in HT-29, 38.0% in LOVO and 84.1% in fibroblasts. At 10^{-4} M, proliferation was further reduced, reaching 0.7% in CC-531, 6.8% in HT-29, 7.0% in LOVO and 1.0% in fibroblasts. In contrast, 10^{-4}M CRA decreased proliferation to 45.2% in CC-531, 68.7% in HT-29, 61.1% in LOVO and 70.0% in fibroblasts. At 10^{-5}M CRA, no significant reduction of proliferation could be determined (98.1% in CC-531, 93.2% in HT-29, 85.9% in LOVO, 94.9% in fibroblasts). Details are given in Table I.

ADA induces apoptosis more effectively than CRA

FACS analysis revealed a time- and dose-dependent induction of cell death in all tested cell lines. For 10^{-4} M ADA, apoptosis began to increase after 18 hr and reached its maximum after 48 or 72 hr, with apoptosis levels of 74.1% in CC-531, 48.8% in HT-29 and 64.1% in LOVO. Induction of apoptosis was less distinct after treatment with 10^{-5} M ADA, reaching a maximum after 72 hr, with 26.4% in CC-531, 40.9% in HT-29 and 32.8% in LOVO. By comparison, incubation with CRA (10^{-4} M) induced lower apoptosis rates, reaching 41.0% in CC-531, 7.1% in HT-29 and 10.0% in LOVO after 72 hr. No increase of apoptosis rate could be detected for concentrations $\leq 10^{-5}$ M CRA (Table II, Fig. 2). Control cells were significantly less affected, displaying no elevated apoptosis rate, when treated with CRA or 10^{-5} M ADA and only moderate apoptosis rates when treated with 10^{-4} M ADA. Apoptosis was confirmed by immunofluorescence staining of cells treated with 10^{-4}M ADA for 24 hr.

Activity of caspase-3 is increased by ADA

Activity of caspase-3 (given as a ratio *vs.* untreated cells) rose 2.6-fold in CC-531, 1.9-fold in HT-29 and 2.4-fold in LOVO but

TABLE I – REDUCTION OF PROLIFERATION RATE IN COLORECTAL CANCER CELL LINES AND HUMAN FORESKIN FIBROBLASTS AFTER 24 HR INCUBATION WITH INCREASING CONCENTRATIONS OF ADA AND CRA

	ADA			CRA		
	10^{-6} M	10^{-5} M	10^{-4} M	10^{-6} M	10^{-5} M	10^{-4} M
CC-531	110.0 ± 29.1	24.6 ± 3.2*	0.7 ± 0.8*	100.0 ± 1.2	98.1 ± 5.3	45.2 ± 1.8*
HT-29	108.2 ± 16.3	56.0 ± 7.7*	6.8 ± 0.9*	102.1 ± 9.5	93.2 ± 10.8	68.7 ± 7.4*
LOVO	95.7 ± 11.0	38.0 ± 6.7*	7.0 ± 1.1*	89.3 ± 14.7	85.9 ± 1.4	61.1 ± 4.0*
Fibroblasts	84.4 ± 6.7	84.2 ± 9.7	1.0 ± 0.6*	99.7 ± 7.4	94.9 ± 5.7	70.0 ± 3.7*

Growth inhibition was determined by BrdU-incorporation ELISA. Values represent percent remaining proliferation compared to untreated cells. Values are means ± SD of 5 independent experiments performed in triplicate.–*$p < 0.01$ *vs.* untreated controls.

TABLE II – INCREASED RATE OF APOPTOSIS IN CC-531, LOVO AND HT-29 COLON CANCER CELLS AND HUMAN FORESKIN FIBROBLASTS AFTER TREATMENT WITH INCREASING CONCENTRATIONS OF ADA AND CRA FOR 3–72 HR

Time (hr)	Untreated	ADA		CRA	
		10^{-5} M	10^{-4} M	10^{-5} M	10^{-4} M
CC-531					
3	3.38 ± 1.37	3.84 ± 1.66	6.32 ± 2.75	n.d.	3.46 ± 1.21
6	3.18 ± 1.03	4.14 ± 1.92	10.90 ± 1.32	n.d.	3.29 ± 1.44
12	4.60 ± 0.79	6.92 ± 0.73	24.30 ± 8.89	n.d.	5.85 ± 2.47
18	4.22 ± 1.35	6.93 ± 1.74	18.09 ± 2.30	n.d.	16.21 ± 4.31
24	3.69 ± 0.26	5.39 ± 0.85	73.06 ± 0.25	3.17 ± 0.90	22.73 ± 11.7
48	2.91 ± 0.22	10.75 ± 0.20	79.97 ± 0.48	4.67 ± 0.67	35.10 ± 0.71
72	3.55 ± 0.21	26.37 ± 1.50	74.09 ± 1.58	4.82 ± 0.35	41.00 ± 5.09
HT-29					
3	1.81 ± 0.35	2.67 ± 0.41	6.61 ± 0.69	1.96 ± 0.37	3.02 ± 0.48
6	1.09 ± 0.09	1.27 ± 0.43	10.27 ± 0.68	4.36 ± 1.8	3.88 ± 1.19
12	1.62 ± 0.35	2.82 ± 1.02	14.01 ± 2.25	8.12 ± 1.37	10.29 ± 2.37
18	1.91 ± 0.88	3.36 ± 2.90	12.49 ± 6.28	5.84 ± 1.14	8.15 ± 0.27
24	1.54 ± 0.46	3.35 ± 0.45	17.49 ± 0.72	4.3 ± 0.40	2.61 ± 0.37
48	1.96 ± 0.67	22.06 ± 1.70	68.07 ± 5.09	2.09 ± 1.00	2.27 ± 0.44
72	2.51 ± 1.67	40.85 ± 5.00	68.83 ± 0.88	2.09 ± 4.11	2.27 ± 0.48
LOVO					
3	1.51 ± 0.64	0.77 ± 0.06	4.97 ± 0.04	n.d.	n.d.
6	2.02 ± 0.37	3.59 ± 3.17	12.48 ± 4.54	n.d.	n.d.
12	1.00 ± 0.02	2.87 ± 1.74	8.08 ± 1.93	n.d.	n.d.
18	1.26 ± 0.73	2.44 ± 0.80	7.84 ± 0.74	4.21 ± 1.32	4.74 ± 0.79
24	1.18 ± 0.32	4.49 ± 1.26	16.76 ± 1.14	4.50 ± 0.67	6.33 ± 1.87
48	0.85 ± 0.05	21.48 ± 4.67	65.98 ± 5.60	7.10 ± 2.35	10.40 ± 1.78
72	3.15 ± 0.47	32.77 ± 1.56	64.05 ± 1.41	7.10 ± 1.45	10.40 ± 0.40
Fibroblasts					
3	2.41 ± 0.46	7.72 ± 0.50	23.66 ± 0.11	1.00 ± 2.00	1.00 ± 2.00
6	1.21 ± 0.67	2.73 ± 1.42	13.78 ± 3.32	2.00 ± 1.00	1.00 ± 1.00
12	3.32 ± 2.59	4.76 ± 5.00	22.53 ± 4.83	2.80 ± 1.25	7.10 ± 2.64
18	3.81 ± 0.43	3.54 ± 1.60	16.47 ± 1.34	3.00 ± 1.00	4.00 ± 2.00
24	1.91 ± 0.35	1.73 ± 1.33	19.85 ± 1.82	4.00 ± 4.00	5.00 ± 1.00
48	2.97 ± 0.57	2.31 ± 0.13	21.96 ± 7.16	4.00 ± 2.00	8.00 ± 9.00
72	2.35 ± 1.16	5.51 ± 1.5	47.71 ± 8.70	4.00 ± 3.00	7.00 ± 5.00

Apoptosis rate was determined by measuring sub-G_1 events in FACS analysis after propidium iodide staining of cells. Values are means ± SD of 3 independent experiments. n.d., not determined.

remained low in fibroblasts (1.3-fold increase *vs.* untreated controls) after 24 hr of incubation with 10^{-4} M ADA. In contrast, activity of caspase-8 remained relatively unaffected in control fibroblasts (Fig. 3). Raji cells showed a 3.3-fold increase of caspase-8 activity after incubation with 10^{-5} M dexamethasone for 24 hr.

ADA disturbs $\Delta\Psi_M$

JC-1 staining showed in CC-531 cells that the percentage of red fluorescence (as a correlate for intact $\Delta\Psi_m$) decreased compared to untreated cells by 17.2%, 16.8% and 44.5% (10^{-6}, 10^{-5} and 10^{-4}M ADA after 24 hr, respectively) (Fig. 4). In HT-29 cells, the effects were similar (8.9%, 17.8% and 49.4%, respectively); in LOVO cells, a less, nonsignificant decrease of red fluorescence was determined. The percentage of red fluorescence in fibroblasts was lower than in CC-531 and HT-29 cells but higher than in LOVO cells.

These findings were correlated with the extent of apoptosis in these cell lines ($r = -0.57$), confirming that disruption of $\Delta\Psi_m$ is involved in apoptosis induction. JC-1 appears to serve as an early marker of apoptosis in the tested cell lines.

ADA induces apoptosis via *the Bax/Bcl-2 pathway*

In ADA-sensitive tumor cells, Western blot analysis showed upregulation of proapoptotic molecules. In these cells (CC-531, HT-29 and LOVO), proapoptotic bax was upregulated or highly expressed and remained high for 6–24 hr. Simultaneously, levels of antiapoptotic bcl-2 decreased. Therefore, the ratio bax:bcl-2 shifted toward proapoptotic bax in ADA-treated cells. Caspase-3 expression was elevated in all examined tumor cell lines.

In contrast, caspase-3 levels remained low in fibroblasts, showing only a minor increase. While bcl-2 remained constant here, levels of proapoptotic bax were diminished after 24 hr, shifting the bax:bcl-2 ratio to antiapoptotic bcl-2. Representative examples of immunoblotting results are shown in Figure 5.

DISCUSSION

Retinoids could be of potential interest as chemopreventive or chemotherapeutic agents in the treatment of malignancies

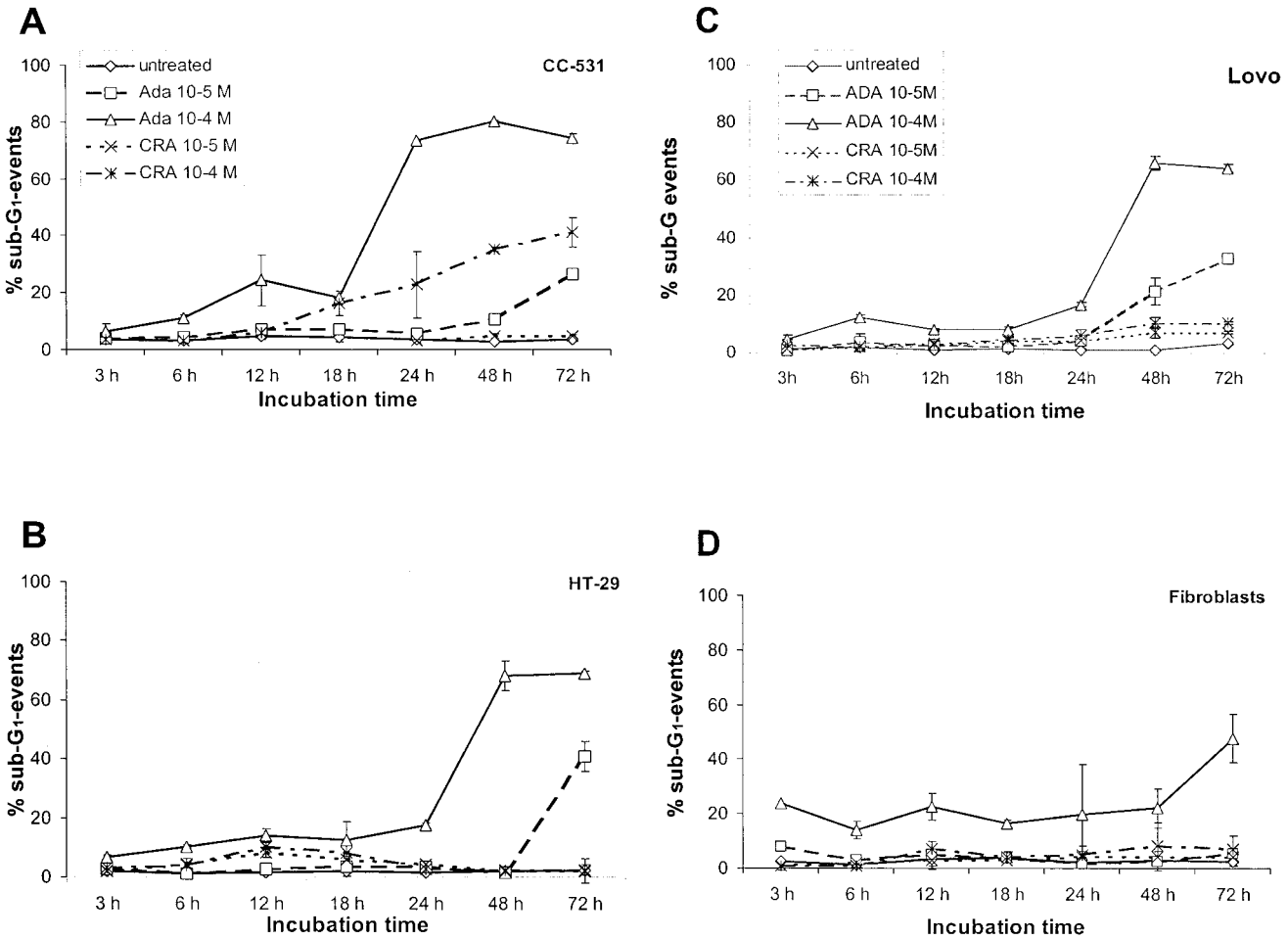

FIGURE 2 – Induction of apoptosis by ADA is time- and dose-dependent. Shown are apoptosis rates measured after incubation with ADA or CRA at 10^{-4} or 10^{-5} M over 3–72 hr. Values are means ± SD of 3 independent experiments.

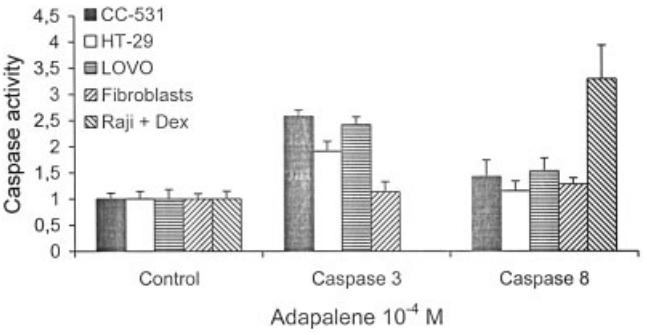

FIGURE 3 – Activity of caspase-3, but not of caspase-8, is elevated in ADA-treated cells. Caspase activity was measured in CC-531, HT-29, LOVO and human foreskin fibroblasts after 24 hr incubation with 10^{-4} M ADA. Results for untreated cells were set at 1.0. Values are means ± SD of 5 independent experiments. Raji cells were incubated with 10^{-5} M dexamethasone for 24 hr.

because of their well-known cell differentiating and potent antiproliferative effects.[18–22] Although there is some evidence that colon cancer cells are sensitive to treatment with natural retinoid derivatives, such as 9- or 13-*cis*-retinoic acid,[2,23–26] there is no information on the effect of synthetic retinoids on colorectal cancer cells. Here, we show that exposure of CC-531, HT-29 and LOVO colon cancer cells to the synthetic retinoic acid ADA causes a rapid decrease in newly synthesized DNA. Furthermore, ADA potently induces apoptosis in these cells in a time- and dose-dependent way. Furthermore, ADA is significantly superior to the natural CRA. Any observed effects were less pronounced in nontransformed fibroblasts, which served as controls. Since primary colon epithelial cells are unstable in cell culture, the effects of ADA on these will have to be investigated in an *in vivo* model.

HT-29 and LOVO colon cancer cells are not responsive to growth inhibition by CRA.[26,27] However, in our study, CRA showed only moderate antiproliferative and proapoptotic effects at 10^{-4} M. CRA was not effective at 10^{-5} M and over 10 times less potent than ADA.

The differences in the effects of CRA and ADA on cell growth and apoptosis induction may be explained by their different metabolic pathways. CRA is bound to a retinol-binding protein and taken up by the target tissue in a process that is not clearly understood.[19] Intracellularly, CRA interacts with the CRBP I/II and CRABP I/II proteins, through which the biologic activity is mediated.[19] CRA is then translocated to the nucleus, where it interacts with the different subtypes of RAR and RXR. The binding affinity of CRA is best for RXRα.[3,6] Upon ligand binding, RAR and RXR isoforms can form homo- or heterodimers that lead to DNA binding of RAREs or RXREs embedded in promoters of retinoid-regulated genes.[3]

In contrast, ADA does not bind to CRABP I or II, which may contribute to the improved *in vivo* tolerance of this compound.[7]

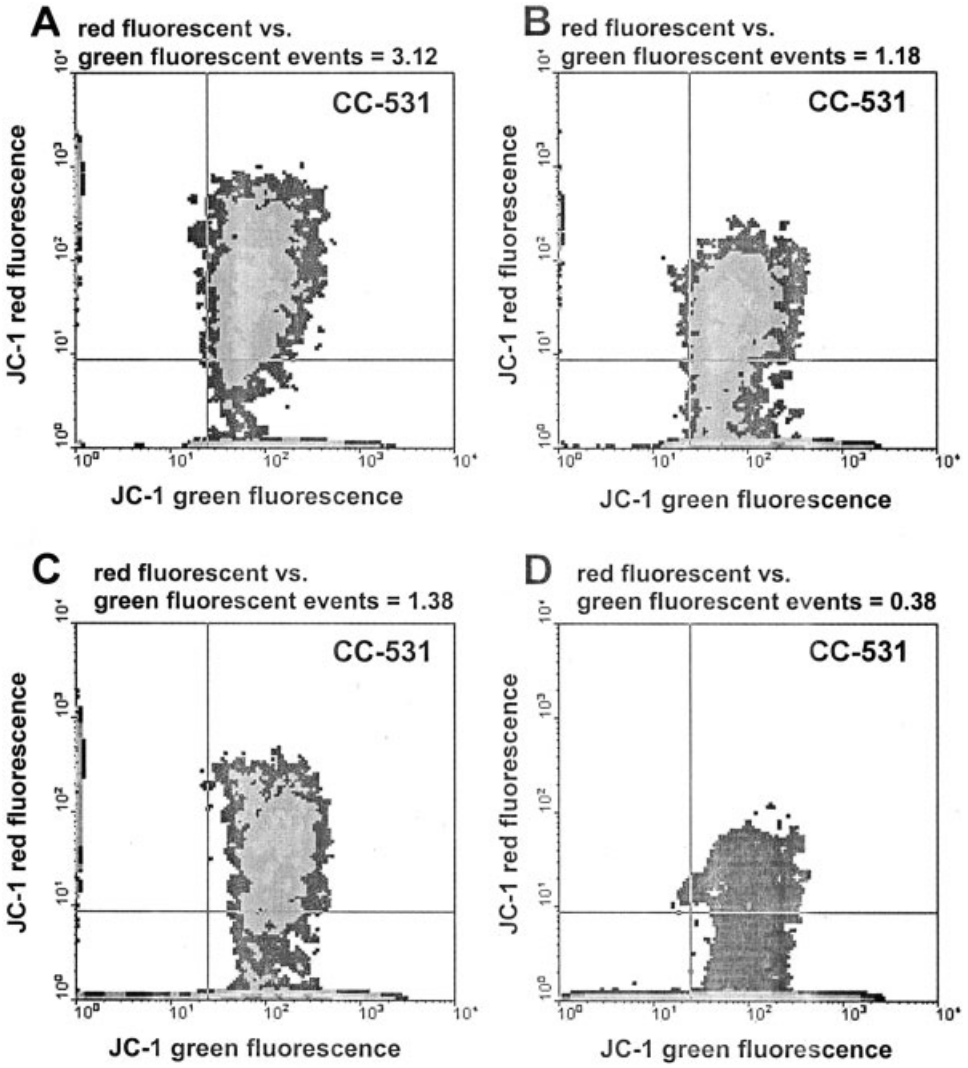

FIGURE 4 – Breakdown of $\Delta\Psi_m$ in CC-531 colon cancer cells. Shown are representative density plots of fluorescence detector FL-1 (green fluorescence) *vs.* FL-2 (red fluorescence), depicting all acquired events. Red fluorescence, intact $\Delta\Psi_m$; green fluorescence, breakdown of $\Delta\Psi_m$. *(a)* Untreated CC531 cells. *(b)* Incubation for 24 hr with ADA 10^{-6}M. *(c)* Incubation for 24 hr with ADA 10^{-5}M. *(d)* Incubation for 24 hr with ADA 10^{-4}M.

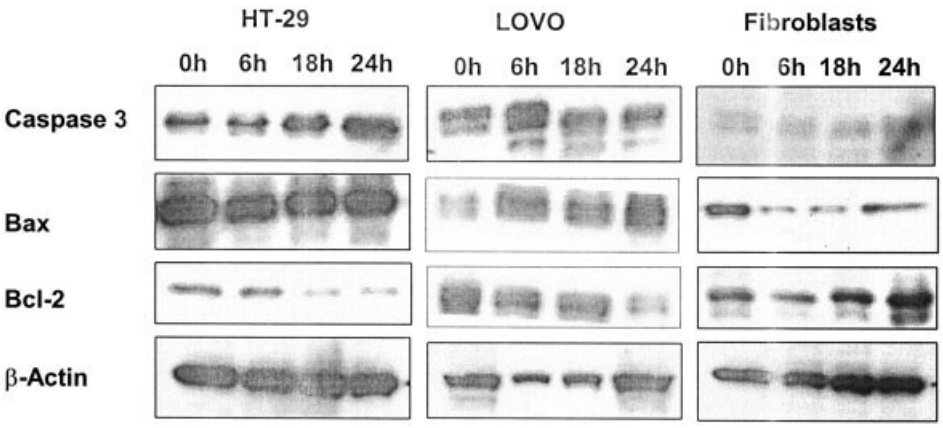

FIGURE 5 – Western blot analysis of HT-29 and LOVO colon cancer cells and human foreskin fibroblasts treated with 10^{-4} M ADA for 0, 6, 18 and 24 hr. Samples were probed with antibodies against caspase-3, bax, bcl-2 and β-actin as an internal standard to show equal loading of lanes.

ADA has high affinity for RARβ and RARγ and only weak affinity for RARα but does not bind to members of the RXR family.[6] While RARα is an inducer of differentiation in leukemia cells,[3] activated RARβ and RARγ are responsible for the epithelial effects of retinoids. RARβ in particular has been described to mediate the chemopreventive actions of retinoids and to transrepress AP-1 signaling. The AP-1 complex is mainly composed of heterodimers of c-Jun and c-Fos; is activated via phosphorylation by MAPKs, *e.g.*, ERK, MAPK and JNK; and binds to control elements present in promoters of genes regulating cell differentiation and proliferation,[28] *e.g.*, matrix metalloproteases,[29] growth factors and inflammatory mediators.[6] ADA is a more potent inhibitor of AP-1 than retinoic acid.[6]

Ligand-specific studies have shown that downregulation of bcl-2 is mediated by RAR and not RXR.[30] Downregulation of bcl-2 leads to breakdown of the $\Delta\Psi_m$, which in turn leads to release of cytochrome *c* and activation of the caspase cascade.[31–34] Upregulation of proapoptotic bax is a requisite gateway to mitochondrial dysfunction.[35] Our results indicate that ADA induces apoptosis by recruiting the mitochondrial pathway of caspase activation by shifting the bax:bcl-2 ratio toward proapoptotic bax. Several models of the proapoptotic properties of members of the bcl-2 family are currently under discussion: pore formation and release of cytochrome *c*, direct activation of caspases via adaptor molecules and interaction with other mitochondrial proteins like the voltage-dependent anion channel leading to disturbance of mitochondrial homeostasis or formation of weakly selective ion channels.[32,36] These effects lead to a changed electrochemical gradient, which we evidenced by changes in JC-1 red fluorescence, which may serve as an early marker for apoptosis.[15]

Besides these classic pathways of apoptosis induction, retinoids trigger alternative pathways of programmed cell death, *e.g.*, translocation of TR3/NGFIB/Nu77 from the nucleus to mitochondria with subsequent release of cytochrome *c* or activation of $p38^{MAPK}$.[37–39]

We have shown here that ADA stimulates the effector caspase-3, while activation of the initiator caspase-8, which is involved in cell membrane–triggered apoptosis (*e.g.*, via tumor necrosis factor or Fas Ligand),[37] was only limited. These findings further support our observation that induction of apoptosis by ADA is mainly mediated via the mitochondrial pathway of caspase activation. In addition, levels of the CDK inhibitor $p21^{waf1/cip1}$ were not affected by ADA treatment, indicating that inhibition of cyclin–CDK complexes is of minor importance to apoptosis induction by ADA.

In addition to its superior ability to induce proliferation inhibition and apoptosis *in vitro*, ADA may offer several advances over conventional retinoic acid derivatives *in vivo.* It possesses stronger anti-inflammatory properties than other retinoids *in vitro* and *in vivo* due to the inhibition of lipoxygenase pathways.[4–7] It has a sustained comedolytic effect in the rhino mouse model and is antiproliferative *in vitro* and *in vivo.*[6] Due to its chemical structure, ADA exhibits a 5-fold greater light stability than natural retinoids.[7] Its toxicity profile is also more favorable than that of other retinoids; the oral LD_{50} is >5 g/kg in rats and mice, which is higher than that of CRA. Even great amounts of orally administered ADA do not cause any neurologic, hematologic, cardiovascular or respiratory side effects (Galderma information).

In conclusion, ADA is a well-tolerated synthetic retinoid, which appears to be superior to CRA in regard to its safety profile and proapoptotic and antiproliferative effect on human colorectal cancer cells *in vitro.* ADA may contribute to the adjunct therapy of advanced stages of colorectal cancer.

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