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9-AAA inhibits growth and induces apoptosis in human melanoma A375 and rat prostate adenocarcinoma AT-2 and Mat-LyLu cell lines but does not affect the growth and viability of normal fibroblasts

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Abstract. The present study found that, similarly to 5-fluorouracil, low concentrations (1-10 μM) of 9-aminoacridine (9-AAA) inhibited the growth of the two rat prostate cancer AT-2 and Mat-LyLu cell lines and the human melanoma A375 cell line. However, at the same concentrations, 9-AAA had no effect on the growth and apoptosis of normal human skin fibroblasts (HSFs). The differences between the cellular responses of the AT-2 and Mat-LyLu cell lines, which differ in malignancy, were found to be relatively small compared with the differences between normal HSFs and the cancer cell lines. Visible effects on the cell growth and survival of tumor cell lines were observed after 24-48 h of treatment with 9-AAA, and increased over time. The inhibition of cancer cell growth was found to be due to the gradually increasing number of cells dying by apoptosis, which was observed using two methods, direct counting and FlowSight analysis. Simultaneously, cell motile activity decreased to the same degree in cancer and normal cells within the first 8 h of incubation in the presence of 9-AAA. The results presented in the current study suggest that short-lasting tests for potential anticancer substances can be insufficient; which may result in cell type-dependent differences in the responses of cells to tested compounds that act with a delay being overlooked. The observed differences in responses between normal human fibroblasts and cancer cells to 9-AAA show the requirement for additional studies to be performed simultaneously on differently reacting cancer and normal cells, to determine the molecular mechanisms responsible for these differences.

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Introduction

In a previous study, low concentrations of 9-aminoacridine (9-AAA) were observed to effectively decrease the threshold of the direct current electric field strength required for cell electroporation, but did not affect normal and cancer cell viability when applied for 2-3 h (1). Nevertheless, when normal and cancer cells were left to grow in the presence of 9-AAA for 2-5 days, the rat prostate cancer cells and human melanoma cells died, whereas normal human skin fibroblasts (HSFs) continued to grow (1).

The drug 9-AAA and its derivatives have been studied since the 1960's, and have been shown to exhibit a broad spectrum of biological activity. At the beginning of the 20th century, these compounds were applied in medicine against protozoan infections and diseases caused by bacteria and yeasts (2-4). In the second half of the century, studies recognized the mutagenic activity of 9-AAA and its derivatives, in particular in bacteria and yeasts, and have since been extensively studied (5-8). The biological activity of the compounds was previously observed to be associated, among other effects, with their capacity to intercalate into DNA (9). 9-AAA has also been found to be useful in research concerning ion channels in biological membranes (10-12) and has been shown to improve the banding patterns of human and plant chromosomes for image analysis (13). Furthermore, 9-AAA has been applied for analysis of the surface electric potential on surfaces of cells, protoplasts and liposomes (14-17).

A previous study reported that 9-AAA inhibits the growth of animal cells and demonstrates anticancer activity *in vivo* (18). In 1969, Mendecki *et al* reported that 9-AAA inhibited the synthesis of RNA in regenerating rat liver cells grown *in vitro* (19). In general, 9-AAA is applied for a short time at relatively high concentrations (>10 μM), and the effects are observed for a short time (usually <48 h). In the majority

of modern molecular studies concerning the effects of 9-AAA on cancer cells, the effects of 9-AAA were followed for 1-2 days (19-25).

Certain studies have shown that cell responses to external factors are often delayed and become visible after a few days (26,27). In contrast to the majority of reported research, the present study therefore examined the effects of 9-AAA on the growth of 3 cancer cell lines (2 prostate cancer cell lines differing in malignancy and 1 human malignant melanoma) and on normal HSFs in cell culture. The tested 9-AAA was present continuously in the cell culture medium at concentrations that did not significantly affect the viability of cells during the first 8 h of its application. The effects of 9-AAA were compared with the 5-fluorouracil (5-FU), a known anticancer drug that is commonly used for cancer therapy in clinics (28,29).

Materials and methods

Cell cultures. All experiments were performed with normal HSFs and 3 cancer cell lines, including human melanoma A375 cells and 2 rat prostate cancer cell lines from the Dunning R-3327 system: Highly malignant Mat-LyLu and moderately malignant AT-2 (1,30,31). The cells were plated in 6-well Falcon culture plates at a density of 20 000 cells per well, 24 h prior to the addition of 9-AAA or 5-FU (Sigma-Aldrich, St. Louis, MO, USA). HSFs and A375 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose concentration (4,500 mg/l; Sigma-Aldrich), and rat prostate adenocarcinoma AT-2 and Mat-LyLu cell lines were grown in RPMI-1640 medium (Lonza Group, Basel, Switzerland). The two media were supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and with a 1% antibiotics solution at final concentration of 100 international units penicillin, 100 μg streptomycin and 0.25 μg amphotericin per ml (Gibco; Thermo Fisher Scientific, Inc.). The cells were propagated at 37˚C in humid air with 5% CO_2. The tested inhibitors, 9-AAA and 5-FU, were dissolved in cell culture medium and applied in concentrations in the range 1-30 μM, as shown in the Results section. Cells cultured in DMEM alone were used as the control. To estimate the effect of the tested compounds on cell growth, the cells were harvested after 24, 48, 72 and 96 h by trypsinization, washed in phosphate-buffered saline (PBS) by centrifugation at 400 x g and counted using a Bürker haemocytometer.

Cell motile activity. Cell motile activity was examined by recording the movements of individual cells and analyzing cell trajectories, as described in detail in previous studies (30-32). The results are presented in the form of circular diagrams, correlation diagrams and the results of calculations.

Cell viability. Cell viability was tested using trypan blue exclusion tests as described previously (32), and in each sample at least 300 cells were analyzed. The type of cell death, apoptosis or necrosis, was examined with two complementary methods.

An analysis of the apoptotic/necrotic cell death of AT-2 cells, Mat-LyLu cells and HSFs growing in the control media or in the presence of 1, 5 or 10 μM 9-AAA or 5, 10 or 15 μM 5-FU was performed following 24, 48 or 72 h of cell culture using two methods. For the first method, the cells were harvested by trypsinization, washed in PBS and stained with propidium iodide (50 μg/ml; Sigma-Aldrich). The cells were analyzed with a FlowSight image flow cytometer and Ideas 5.0 software (Amnis Corporation, Seattle, WA, USA). For each sample, images of 10,000 single cells were analyzed at 488 and 785 nm light wavelength using three channels, as described previously (33). For the second method, the cells were fixed in 3.5% formaldehyde for 20 min. The cells were then stained in 0.5 ml Hoechst 33342 in PBS (1 μg/ml), prior to direct observation under a fluorescence microscope (Leica DMI6000B, type AF7000; Leica Microsystems GmbH, Wetzlar, Germany) at a light excitation wavelength of 352 nm. For each sample, >250 cells were observed and analyzed. The number of apoptotic cells with fragmented nuclei was counted.

Statistical analysis. All experiments were conducted in triplicate, giving similar results, and data are presented as the mean values. For analysis of cell motility, a Mann-Whitney test was used and results were considered significant if $P<0.05$ (n=50). For other analyses, a two-sample independent Student's t-test was used and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell type-specific effects of 5-FU and 9-AAA upon growth of cancer and normal cells in vitro. In the first series of experiments, the effect of the continuous presence of 9-AAA in cell culture medium, at concentrations of 1-20 μM, on cell proliferation was examined. The effect of 9-AAA on cell proliferation was compared with the effect of 5-FU, a known and commonly used cytostatic drug in clinical oncology, which was applied at concentrations of 5-30 μM. In preliminary experiments (data not shown), 9-AAA and 5-FU, in the examined range of concentrations, did not show acute toxicity, and >90% of all tested cell types survived for 4 and 8 h of incubation in their presence, as tested with the trypan blue exclusion test. The response to these two substances was tested on HSFs, as an example of normal cells, and on 3 neoplastic, established cell lines, including the human melanoma A375, rat prostate highly metastatic adenocarcinama Mat-LyLu and moderately malignant rat prostate AT-2 cell lines (Fig. 1). The highly cell type-specific effects on cell proliferation were recorded.

The examined substances, 5-FU (Fig. 1A-C) and 9-AAA (Fig. 1D-G), in the highest tested concentrations (30 and 20 μM, respectively) had no statistically significant effect upon growth of HSFs. At the concentrations of 5, 10, 15 and 30 μM, 5-FU inhibited proliferation in the two tested rat prostate adenocarcinoma cell lines (Fig. 1B and C; AT-2 P=0.017, P=0.016, P=0.016, P=0.015; MAT-LyLu P=0.031, P=0.029, P=0.027, P=0.025, respectively). At the concentrations of 1, 5, 10 and 15 μM, 9-AAA inhibited cell growth in the tested two rat prostate adenocarcinoma cell lines differing in malignancy (Fig. 1E and F; AT-2 P=0.016, P=0.01, P=0.01, P=0.01; MAT-LyLu P=0.018, P=0.01, P=0.01, P=0.01, respectively). At

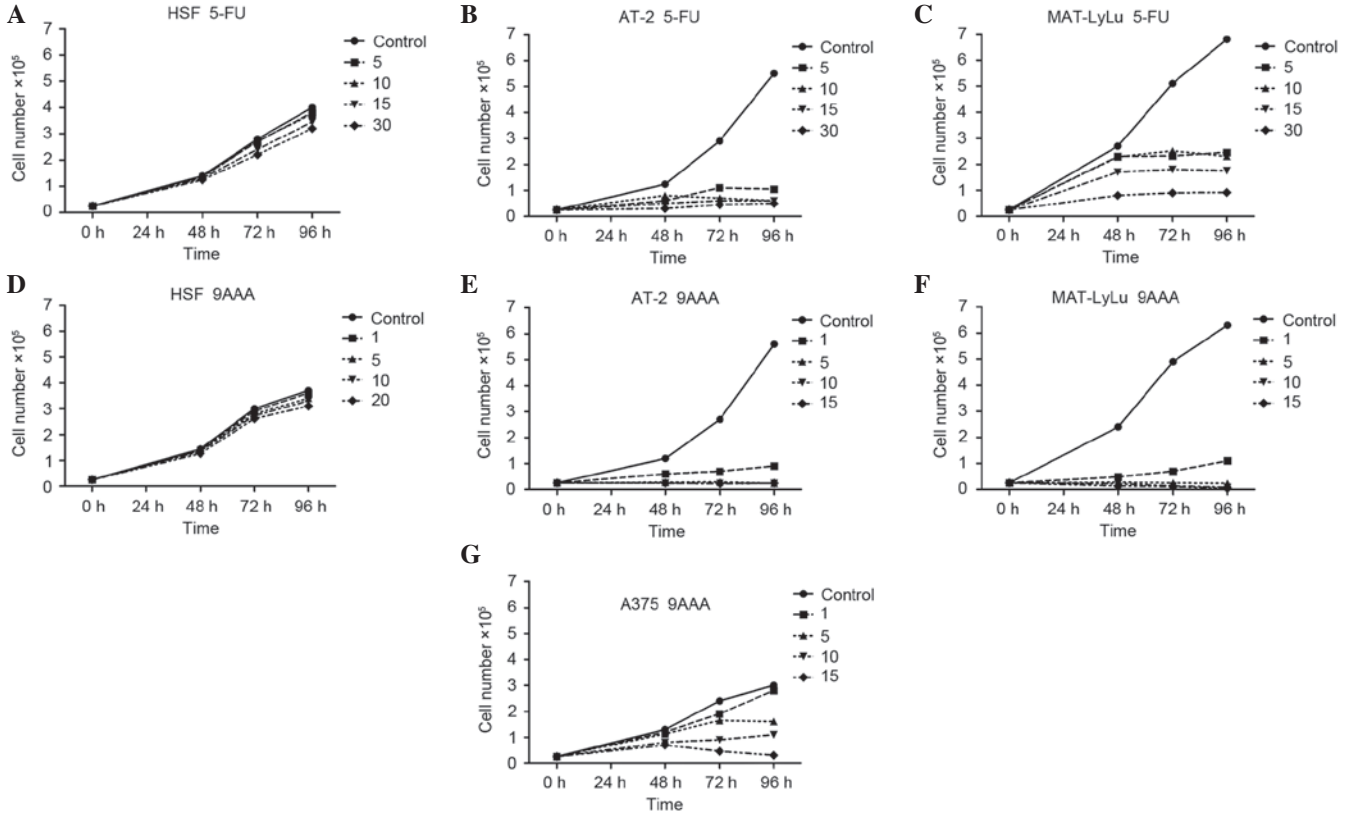

Figure 1. Effects of (A-C) 9-AAA and (D-G) 5-FU on the growth of various cells: (A and D) HSFs; rat prostate adenocarcinoma (B and E) AT-2 and (C and F) Mat-LyLu cell lines; and (G) human melanoma A375 cell line. Abscissa, time of cell growth (h); ordinate, cell number per well (in 6-well culture flasks). 9-AAA, 9-aminoacridine; 5-FU, 5-fluorouracil; HSF, human skin fibroblast.

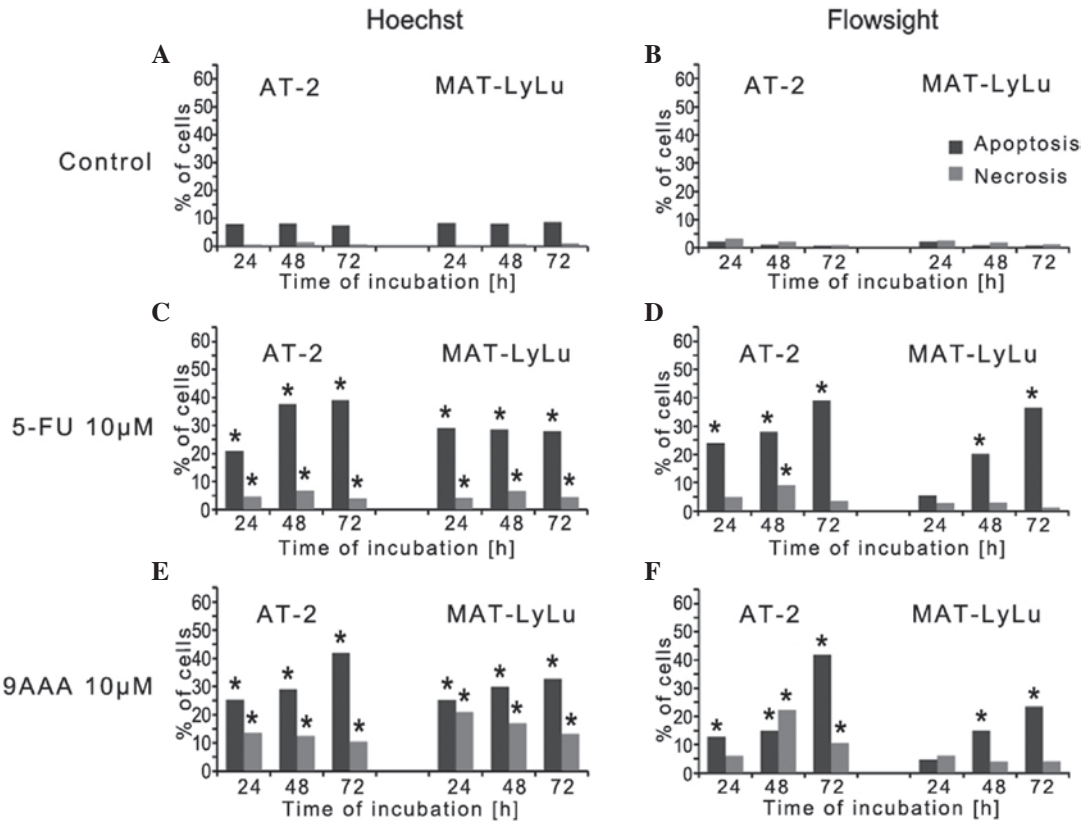

Figure 2. Effects of 10 μM 5-FU or 9-AAA treatment on AT-2 and Mat-LyLu cell death by apoptosis and necrosis, analyzed with two methods. (A and B) Control (no treatment); (C and D) 10 μM 5-FU treatment; (E and F) 10 μM 9-AAA treatment. Results were assessed by (A, C and E) direct observation of cells under fluorescence microscope after Hoechst 33342 staining, and (B, D and F) single cell analysis with FlowSight flow cytometry of propidium iodide-stained cells. 5-FU, 5-fluorouracil; 9-AAA, 9-aminoacridine. Data are expressed as mean values and were analyzed with a two sample independent Student's t-test; $^{*}P<0.05$ vs. control.

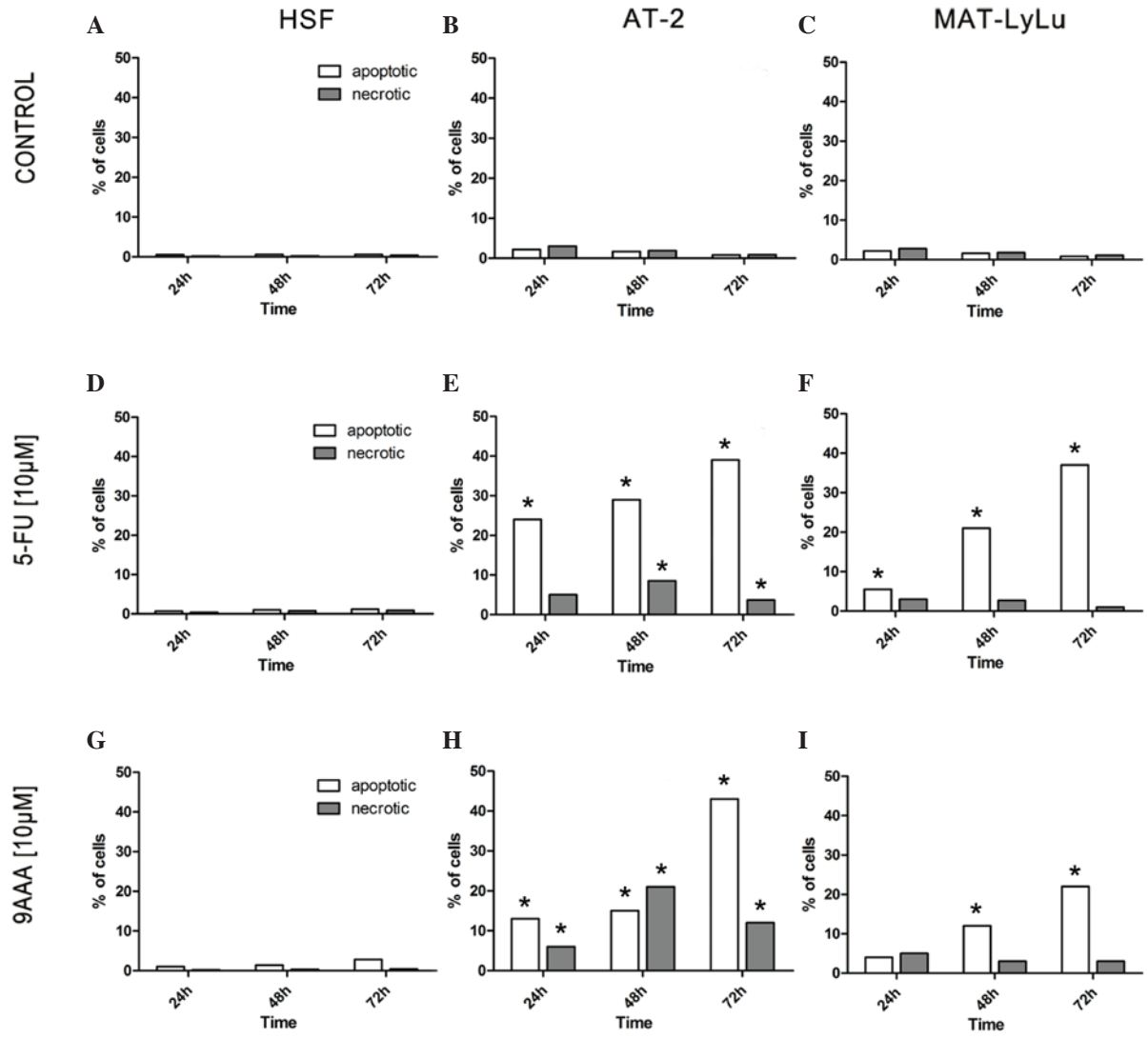

Figure 3. Image FlowSight analysis of (A, D and G) HSF, (B, E and H) AT-2 and (C, F and I) Mat-LyLu cell death by apoptosis and necrosis when growing in control medium or the medium supplemented with 5-FU (10 μM) and 9-AAA (10 μM). HSF, human skin fibroblast; 5-FU, 5-fluorouracil; 9-AAA, 9-aminoacridine. Data are expressed as mean values and were analyzed with a two sample independent Student's t-test; *P<0.05 vs. control.

the lowest tested concentration, 1 μM, 9-AAA fully inhibited the growth of both rat prostate cancer cell lines (P=0.01), but not human melanoma cells. In the A375 melanoma cell culture, 9-AAA inhibited the growth of cells with a delay, when applied at the following concentrations: 5, 10 or 15 μM (Fig. 1G; P=0.034, P=0.031, P=0.019). Notably, the inhibition of neoplastic cell growth caused by 9-AAA and 5-FU was not immediate. Independently, whether the substances were added at the start of the culture or on the second day, the growth inhibition only became marked after 24-48 h incubation of cells in the presence of 5-FU or 9-AAA.

Pro-apoptotic activity of 5-FU and 9-AAA in cancer cells, but not in normal HSFs. The subsequent experiments aimed to examine whether the growth inhibition of the tested cancer cells by 9-AAA is associated with the specific killing of cells by apoptosis or by necrosis. Two methods were applied: i) Cells stained with Hoechst 33342 were directly counted under a microscope to observe the morphology of nuclei in apoptotic cells and necrotic cells. This method permits observation of individual single cells, and therefore is limited to observation of several hundred cells in a sample. ii) In parallel, the samples from the same cell culture were analyzed with the modern method using FlowSight, which analyzes thousands of cells (33). The results obtained using the two methods combined yielded well-corresponding results (Fig. 2).

FlowSight analysis was used to examine the number of cells dying by apoptosis and necrosis in the prescence or absence of 5-FU or 9-AAA (Fig. 3). In HSFs, in the absence of the tested substances, the number of dying cells did not exceed 0.5% (Fig. 3A). In the presence of 10 μM 9-AAA and after 3 days of culture, the number of dying cells increased, but remained <3%, and more cells died by apoptosis compared with necrosis (Fig. 3D and G). By contrast, the cells of the 2 rat prostate cancer AT-2 and MAT-LyLu cells began to die after growing for 24 h in the presence of the tested inhibitors (Fig. 3B, C, E, F, H and I; P=0.01). In the rat prostate cancer AT-2 and Mat-LyLu cell lines, which differ in malignancy, the observed effects of each inhibitor were almost the same. The

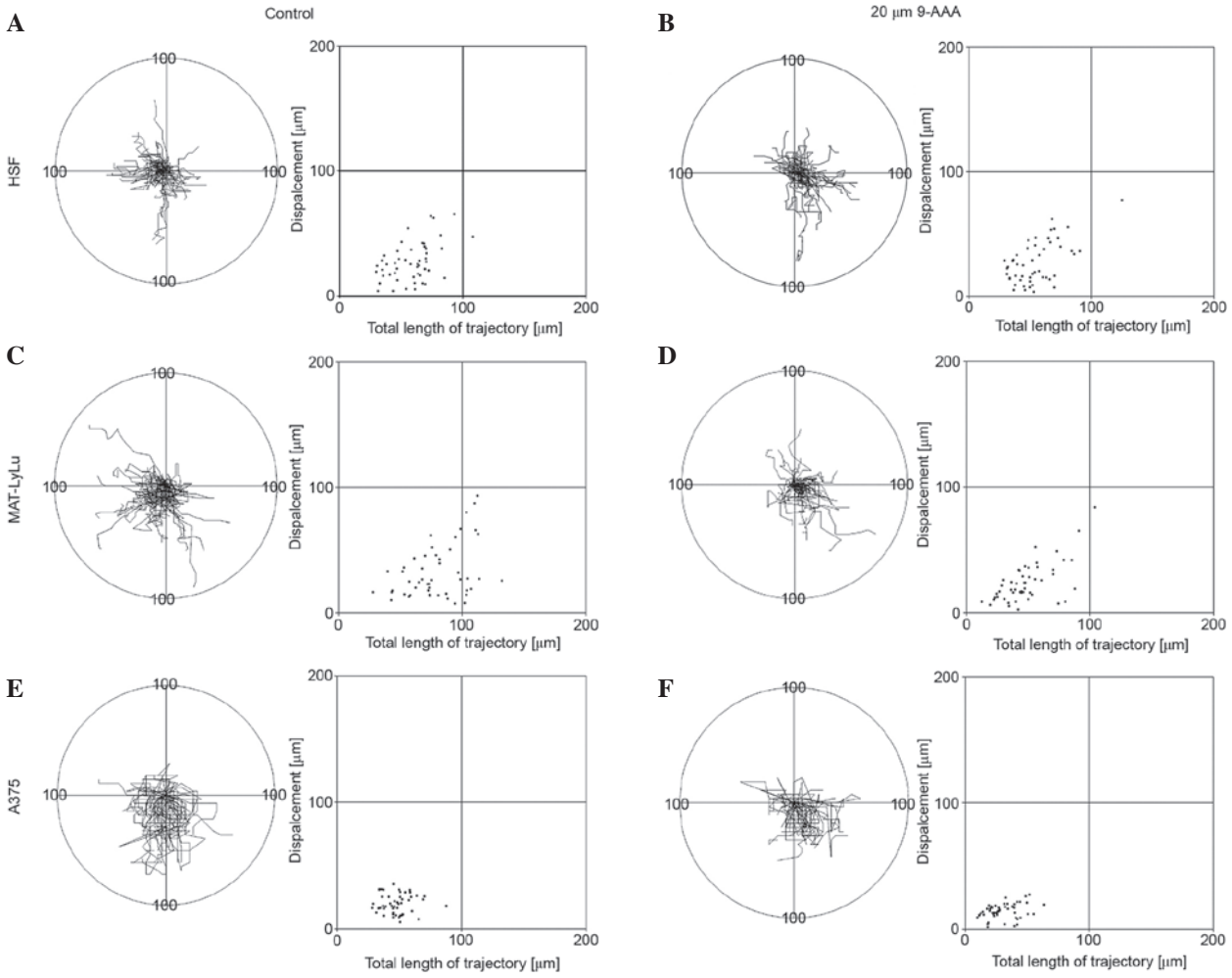

Figure 4. Effect of the medium supplementation with 20 μM 9-AAA on the motile activity of HSFs, rat prostate cancer Mat-LyLu cells and human melanoma A375 cells. The trajectories of HSF migrating (A) under control conditions or (B) in the presence of 20 μM 9-AAA, Mat-LyLu migrating (C) under control conditions or (D) in the presence of 20 μM 9-AAA and A375 cells migrating (E) under control conditions (F) or in the presence of 20 μM 9-AAA are presented in the circular diagrams and in the form of scatter correlation diagrams. Please notice similar decrease in the length of cell trajectory, final translocation and speed of movement in normal and cancer cells. 9-AAA, 9-aminoacridine; HSF, human skin fibroblast.

numbers of dying cells increased with time and after 3 days of cell culture reached >40%. The proportion of cells dying by apoptosis was much greater compared with the cells dying by necrosis. AT-2 cells were found to be slightly more sensitive to the 9-AAA and 5-FU compared with Mat-LyLu. In the presence of inhibitors, despite the great proportion of dying cells, dividing cells were observed via direct observation after staining with Hoechst, in particular, when the inhibitors were applied at the 5 μM concentration.

Lack of differential effects of 5-FU and 9-AAA on cancer and normal cell movement. Since cancer cell malignancy and the capacity of cells to metastasize is usually correlated with cell motile activity, the present study examined the effect of 9-AAA on the migration of 3 cell lines, including normal HSFs, rat prostate malignant cancer Mat-LyLu and human melanoma A375 cells. In previous experiments, a single-cell approach and computer-aided methods were applied for recording and analyzing cell movement trajectories in the absence and in the presence of 9-AAA (30,33). Cell movement was recorded 24 h after cells were seeded. The control experiments were carried out in cell culture medium. The effects of 9-AAA on cell movement were examined at a concentration of 20 μM, the highest examined in experiments concerning cell growth. The results of the cell movement records in the absence or in the presence of the highest concentration tested are shown in Fig. 4. Even at the highest concentration of 9-AAA, which inhibited the growth of human melanoma cells and rat prostate malignant cancer Mat-LyLu cell line (but not the growth of HSFs), the 9-AAA had no statistically significant different effect on the speed of cell movement/length of cell trajectories, the shape of the cell trajectories, or the cell turning frequency of the normal HSF or 2 cancer cell lines (Fig. 4). In all 3 cell lines the presence of 9-AAA in cell medium similarly affected the speed of cell movement and the length of cell trajectories.

Discussion

The results presented in the current study show that 9-AAA and 5-FU, when present at low concentrations (1 or 5 μM, respectively) in cell culture medium, efficiently inhibited the

growth of 2 rat prostate cancer cell lines, AT-2 and Mat-LyLu, but had no influence on the viability and growth of HSFs. Human melanoma A375 cells were inhibited at a concentration of 5, 10 and 15 μM 9-AAA. Even greater concentrations of these substances (20 and 30 μM) did not inhibit growth of the normal HSFs.

The growth inhibition in the examined cancer cell lines was caused by 9-AAA and 5-FU at concentrations that did not affect the cell viability if applied for 8 h. The microscopic observation of cancer cells, for which proliferation was retarded or inhibited after 3 days of cell culture in the presence of 9-AAA or 5-FU, showed dividing cells among cells undergoing death by necrosis or by apoptosis (formation of apoptotic bodies). One can expect that, in heterogeneous cell populations such as populations of cancer cell lines (32,33), the cells that proliferate are present alongside cells that die by apoptosis or necrosis. The present study demonstrated that the inhibition of increased cell numbers in cancer cell cultures in the presence of 5-FU and 9-AAA resulted from an increased proportion of dying to proliferating cells over time.

Therefore, the capacity of 9-AAA and 5-FU to induce apoptosis was compared in normal HSFs and in rat prostate cell lines. The application of two methods that permitted the discrimination of cells dying by apoptosis and necrosis yielded the same results. Both 9-AAA and 5-FU induced an increasing number of cells dying by apoptosis in cancer cell populations, but did not markedly change the proportion of apoptotic cells in normal HSF cultures.

Numerous research concerning the effects of 9-AAA on cancer cells *in vitro* have been reported, however, effects were observed within 3-8 h of 9-AAA administration. The tested substances were applied for a short time and results were followed for a few hours, usually in one type of cancer cell. Nevertheless, among the numerous reports concerning the impact of 9-AAA and its derivatives (for example quinacrine) on cancer cells, certain studies appear to have demonstrated similar results compared with the findings indicated in the present study.

Gurova *et al* (34) reported that 9-AAA pro-apoptotic activity is associated with inhibition of the transition of cells from G1 phase to the S phase of the cell cycle. This may partly explain why the inhibition of cancer cell proliferation in the presence of low concentrations of 9-AAA is observed with delay, only after 24-48 h. In asynchronous cell culture, only a small fraction of cells may be susceptible to the inhibitory activity of the inhibitor in a short window of time. However, there is a lack of studies on this topic of research, which may explain why there are such large differences in the cellular response to low concentrations of 9-AAA or 5-FU between the tested cancer cell lines and normal HSFs. These differences may be associated with the observations made by Wang *et al* (22), Gurova *et al* (34) and Guo *et al* (35), regarding the effects of 9-AAA on proteins, including tumor protein 53, Bcl2 associated X protein and transcription factor nuclear factor-κ B, and/or by Teitelbaum *et al* (36) on topoisomerase II and topoisomerase I (37). Expression of these proteins usually differs in normal and cancer cells (34). However, the explanation of the results observed for cancer cells *in vitro* and *in vivo*, reported by Etchison *et al* (25) and Guo *et al* (35), requires supplementation by results from parallel, simultaneous experiments involving cancer and normal cells, differing in their responses to the tested drugs.

The difference in reactions among various cells could also be partly due to the differences in electrochemical properties of the surface of cancer and normal cells (38-42). As a cationic substance, 9-AAA may more strongly affect the cancer cells, whose surface is more negatively electrically charged compared with the surface of normal cells, as the effect of 9-AAA on cell membranes was previously found to depend upon the negative electric charging of cell surfaces and liposomes (14,16,17,43). This phenomenon may result in varying responses of cells, differing in membrane electric properties. In addition, 9-AAA was reported to affect electric charges on cell surface and ion fluxes across membrane channels (8-12,14-17). This may modify the penetration of 9-AAA into cells or an operation of signaling pathways and interactions among cells. Therefore, the differences between the responses of cancer and normal cells do not appear to result solely and primarily from the often investigated intercalation of 9-AAA to DNA and its mutagenic activity.

It is well documented that the capacity of cancer cells to metastasize correlates with their motile activity (30,44,45). The results of the current demonstrated that 9-AAA and 5-FU similarly affected the movement of cancer and normal cells, at least within the first few hours of its application to the cells. This shows that the agents that can affect cancer cell malignancy and the capacity of cells to metastasize, i.e. influence cell motile behavior, are not necessarily the same agents that can result in the inhibition of cell growth, in particular if they induce cell death by apoptosis.

Teitelbaum *et al* (36) reported that 9-AAA derivatives in micromolar concentration can be efficient against mouse glioblastoma, and Etchison *et al* (25) suggested that the 9-AAA derivatives can be effective against small cell lung cancer. Overall, these observations suggest that 9-AAA and its derivatives may be promising targets for the chemotherapy of types of cancer that are considered to resistant to the majority of the presently applied anticancer drugs (25,36,46).

In conclusion, the results of the present study, which regards the effectiveness of specific cancer cell growth inhibition by 5-FU and 9-AAA, indicate the requirement for additional studies on the molecular mechanisms responsible for the varied responses of normal and cancer cells to 9-AAA and its derivatives, and suggest that short term cytotoxicity evaluation can lead to potential anticancer compounds being overlooked. The results presented show that 9-AAA at low concentrations specifically inhibits growth and induces apoptosis in human melanoma A375 cells and 2 rat prostate cancer cells lines, but has no effect on the survival and growth of HSFs in tissue culture.

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