Eur J Med Res (2006) 11: 313-321

Chemopotentiating Effects of a Novel NAD Biosynthesis Inhibitor, FK866, in Combination with Antineoplastic Agents

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Abstract
FK866 is a novel anticancer agent that was previously shown to interfere with NAD^+ biosynthesis by inhibition of nicotinamide phosphoribosyltransferase and to initiate apoptosis in cancer cells. As NAD^+ is involved in cellular DNA repair processes, the present *in vitro* study on THP-1 and K562 leukemia cells was conducted to investigate the cytotoxicity of FK866 combination treatment with various cytotoxic agents: the antimetabolite Ara-C, the DNA-intercalating agent daunorubicin and the alkylating compounds 1-methyl-3-nitro-1-nitrosoguanidinium (MNNG) and melphalan. Cell viability after drug exposure was assessed by propidium iodide (PI) staining. Non-cytotoxic concentrations of FK866 (10^{-9}M or less), applied simultaneously or 24 hours before adding cytotoxic agents, caused a depletion in the intracellular NAD^+ and – to a lesser extent – NADH levels in THP-1 cells. After 48 and 72 hours treatment with daunorubicin and Ara-C, respectively, increased cell death was observed in THP-1 cells that were pretreated with FK866, as compared to cells exposed to antineoplastic drugs alone. However, this effect was transient, and there was no difference in cell survival after 72 hours incubation with daunorubicin or 96 hours with Ara-C.

Non-toxic concentrations of FK866 added 8, 16, or 24 hours before starting treatment with the PARP-activating agent MNNG synergistically decreased intracellular NAD^+ contents, and increased MNNG-induced cytotoxicity both in THP-1 and K562 cells for at least 72 hours. This effect was less pronounced when FK866 was used in combination with another alkylating agent, melphalan. The PARP inhibitor 3-aminobenzamide delayed MNNG-induced cytotoxicity by 24 hours both in cells that were pretreated with FK866 and in non-pretreated cells. 48 hours later, the protective effect of 3-aminobenzamide could no longer be observed, but FK866-pretreated cells retained increased sensitivity to MNNG.

In conclusion, the chemosensitizing effect of FK866 on cell death induced by antineoplastic drugs was particularly obvious in combination with substances like MNNG that cause NAD^+ depletion *per se*. It was less pronounced and only transiently measurable in combination with daunorubicin, Ara-C, and melphalan, respectively. These results may indicate different levels of DNA damage implicated in the action of the cytotoxic agents used.

Key words: Apoptosis, MNNG, FK866, NAD, Melphalan

Introduction

The main objectives of combination chemotherapy are, on the one hand, an increased response rate against the tumour and, on the other hand, minimization of adverse effects of antineoplastic drugs without compromising efficacy of treatment. Biochemical modulation of anticancer response includes manipulation of intracellular metabolic pathways using certain pharmacological agents to produce selective enhancement of antitumour effects by the anticancer drug [1]. The combination with metabolic inhibitors may also permit dose reduction of conventional cytotoxic drugs, while their biological activity and the antitumour response remain unaltered. This strategy is based on the hypothesis that the neoplastic cell population in a tumour is heterogeneous and contains cancer cells of variable sensitivity to antineoplastic agents [2]. Since energy depletion is a critical biochemical event in cell death, less sensitive cells obviously do not receive enough damage to deplete their ATP content and therefore can survive. Biochemical modulation to further reduce ATP to lethal levels would kill these sub-lethally injured cells. NAD^+ plays an important role in aerobic ATP synthesis as a coenzyme of mitochondrial dehydrogenases that are components of the respiratory chain. Furthermore, DNA damage, that plays a key role in the pharmacodynamic effects of most anticancer drugs, entails activation of poly(ADP-ribose)polymerase (PARP). This enzyme catalyses the breakdown of NAD^+, accompanied by transfer of ADP-ribose moieties on itself and on nuclear proteins such as histones, thus facilitating access for repair enzymes to the sites of DNA damage [3]. The depletion of NAD^+ that follows PARP activation results in lower intracellular ATP levels, since the cells consume ATP in an effort to resynthesize NAD^+ [4]. It has been demonstrated that the NAD^+ analogue 6-aminonicotinamide potentiated cytotoxic and tumoricidal effects of radiochemotherapy [5]. Moreover, Tiazofurin, a synthetic nicotinamide-mononucleotide analog, was

active in patients with refractory acute myeloid leukemia or blast crisis of chronic myeloid leukemia [6]. NAD^+-depleting agents therefore appear to be promising components of therapeutic regimens that may be useful in cancer treatment [7]. FK866 is a potent and specific inhibitor of nicotinamide phosphoribosyltransferase (NAPRT), a key enzyme involved in the biosynthesis of NAD^+ from its precursor nicotinamide [8]. Inhibition of NAPRT leads to depletion of intracellular NAD^+ [9], which in turn may induce cell death. Furthermore, a marked decrease of nicotinamide mononucleotide adenylyltransferase (NMNAT) activity, the other enzyme involved in NAD-synthesis, has been observed in tumour cells, so that healthy tissues may be less affected after treatment with FK866 [7].

This report describes the effects on the viability of THP-1 and K562 cells of non-toxic concentrations of FK866 used in combination with the alkylating substance MNNG and the following standard chemotherapy drugs with different modes of action: daunorubicin, Ara-C, and melphalan. In addition, we investigated the influence of these drugs alone or in combination with *per se* non-toxic concentrations of FK866 on cellular oxidoreductase activity and intracellular NAD^+ levels in THP-1 human leukemia cells.

Materials and Methods

Materials

Melphalan (L-phenylalanine mustard), nicotinamide and daunorubicin were purchased from Sigma; Ara-C from Pharmacia; MNNG (1-methyl-3-nitro-1-nitrosoguanidinium) from Fluka; 3-aminobenzamide (3-AB) from Biomol; propidium iodide from Calbiochem; WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) and 1-methoxy-PMS (1-methoxyphenazine methosulphate) from Serva; NAD^+ from Roche. Stock solutions were made by dissolving drugs in DMSO or DMF and stored at –20 °C. Standard chemicals were purchased from Merck or Sigma.

Cells and Cell Culture

THP-1 and K562 cells, derived from patients with acute monocytic leukaemia (FAB M4), and blast crisis of chronic myeloid leukaemia, respectively, were purchased from the American Type Culture Collection (ATCC Rockville, Maryland, USA). The cells were maintained in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/mL) and streptomycin (100µg/mL) (Biochrom). Cells were cultivated in 5% CO_2 / 95% humidified air at 37 °C. During culture, the cell density did not exceed 10^6 cells/ml. Drugs from stock solutions were added to the cell culture medium resulting in the different concentrations indicated.

WST-1 Assay

Cellular dehydrogenase activity as an indicator of metabolic activity was determined by the WST-1 assay. During drug treatment, the cells were incubated in 96-well plates in a final volume of 100µL; 10µL of WST-1 dye solution (Roche) was added directly to each well at different time points. The plate was shaken and incubated for one hour. Light absorbance at 450 nm was then measured using an ELISA reader (Biotek Instruments). Relative cellular dehydrogenase activity, which is dependent on both, the respective amounts of active enzyme and its substrate NAD, was calculated as the percentage of signal in untreated control cells.

PI Assay

The propidium iodide (PI) assay was used to determine the fraction of dead cells. During cell death, the cytoplasmic membrane potential collapses and PI can freely penetrate the cells eventually staining the nuclei through intercalating into the DNA double strand. In contrast, their intact cytoplasmic membranes exclude PI from viable cells rendering them unstained. Cells treated with drugs were incubated in 96-well plates at a final volume of 200 µL. Cell concentration of THP-1 and K562 cells at the beginning of incubation was adjusted to 40,000/mL. After adding 25µL of PI dye solution at a final concentration of 15 µg/mL to each well, the plate was shaken and incubated for one hour at room temperature in the dark. The fluorescence of PI-permeable (dead) cells was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using an ELISA reader Biotek FL500 (Biotek Instruments). Subsequently, 20µL of 1% Triton X-100 solution was added to each well, and the plate was shaken and incubated for an additional one hour in the dark at room temperature. Fluorescence was then determined to measure PI uptake of the whole population of permeabilized cells (vital+dead). The proportion of dead cells was calculated as a ratio of the PI signal from dead cells and that from Triton X-100 permeabilized cells and expressed as percent PI positive cells.

NAD(H) Determination

One million of logarithmically growing trypan blue negative cells were collected and washed once in PBS.

For the determination of the intracellular NAD^+ or $NADP^+$ 200µl of 0.5M perchloric acid were added to the cell pellet and the lysates were incubated at 4 °C for 15 min. Then 60µl of neutralisation buffer containing 2N KOH, 0.2M K2HPO4/KH2PO4, pH = 7.5, were added, the lysates were centrifuged at 12,500×g for 3 min, clear supernatants were collected and stored at –70°C. For the determination of intracellular NADH or NADPH 100µl of alkaline lysis buffer (0.02N NaOH, 0.5mM L-cysteine) were added to the cell pellet. To reduce the viscosity of the samples, lysates were incubated at 60 °C for 10 min, neutralised with 60µl of 0.25M Gly-Gly buffer, pH = 7.4, centrifuged at 12,500×g for 3 min, clear supernatants were collected and stored at –70 °C.

To determine the intracellular NAD^+/NADH 10µl of cell extracts were pipetted into a 96-well plate and 150µl of reaction mixture containing 60mM Gly-Gly buffer, pH = 7.4, 1.63 mM WST-1, 65.3 µM 1-methoxy-PMS, 60mM nicotinamide, 300mM ethanol and

20U ADH were added. For the measurement of intracellular $NADP^+$/NADPH 150µl of cycling reagent containing 50mM Tris, pH = 8.1, 1.63 mM WST-1, 65.3 µM 1-methoxy-PMS, 5mM glucose-6-phosphate and 0.45U G6PDH were added to 10µl of cell extract.

The plates were incubated for 40 min at 37 °C and the absorbance was measured using an ELISA-reader (Biotek instruments, Neufahrn, Germany) at the wavelength of 450 nm. The intracellular NAD^+/NADH/NADPH content was calculated using a calibration curve measured with external standards.

Statistical Analysis

The Student's T test was used to determinate statistical significance. The level of significance was defined as $p<0.05$.

Results

Cytotoxic and NAD(H)-depleting Effects of FK866 in THP-1 Cells.

First we examined the cytotoxicity of FK866 in THP-1 cells using the PI assay 24, 48, and 72 hours after begin of treatment. As illustrated in Figure 1A, concentrations of FK866 from 10^{-11} M to 10^{-8} M were used. We could not demonstrate any toxicity 24 hours after starting treatment with FK866. Cell death was only observed, if concentrations above 3×10^{-10} M FK866 were used for at least 48 hours. Then we examined the effects of FK866 on the function of NAD^+-dependent dehydrogenases in THP-1 cells. Using the WST-1 test, we found that treatment of THP-1 cells with 10^{-10} M FK866 for 19 hours caused a two-fold decrease of cellular reduction capacity, as compared to control cells. The reduction of WST-1 dropped by 65-75 %, when higher concentrations of FK866 were used (Fig. 1B). Further, we determined the intracellular NAD^+ and NADH contents after 17 hours exposure of THP-1 cells to different concentrations of FK866. The resulting NAD^+ content in cells treated with 10^{-10} M FK866 was only about one third the amount of control cells (Fig. 1C). The effect of the same FK866 concentration on the intracellular NADH levels was less pronounced. The NADH content of drug treated cells was still above 50% of control cells (not shown). After incubation of THP-1 cells with 10^{-9} M FK866 for 17 hours 90% and 40% decrease in intracellular NAD^+ and NADH levels, respectively, could be observed.

Effect of FK866 on Cell Death Induced by Daunorubicin in THP-1 Cells

The effect of daunorubicin on the induction of cell death in THP-1 cells alone or in combination with FK866 was examined using the PI test. For combination experiments 3×10^{-10} M FK866 was added simul-

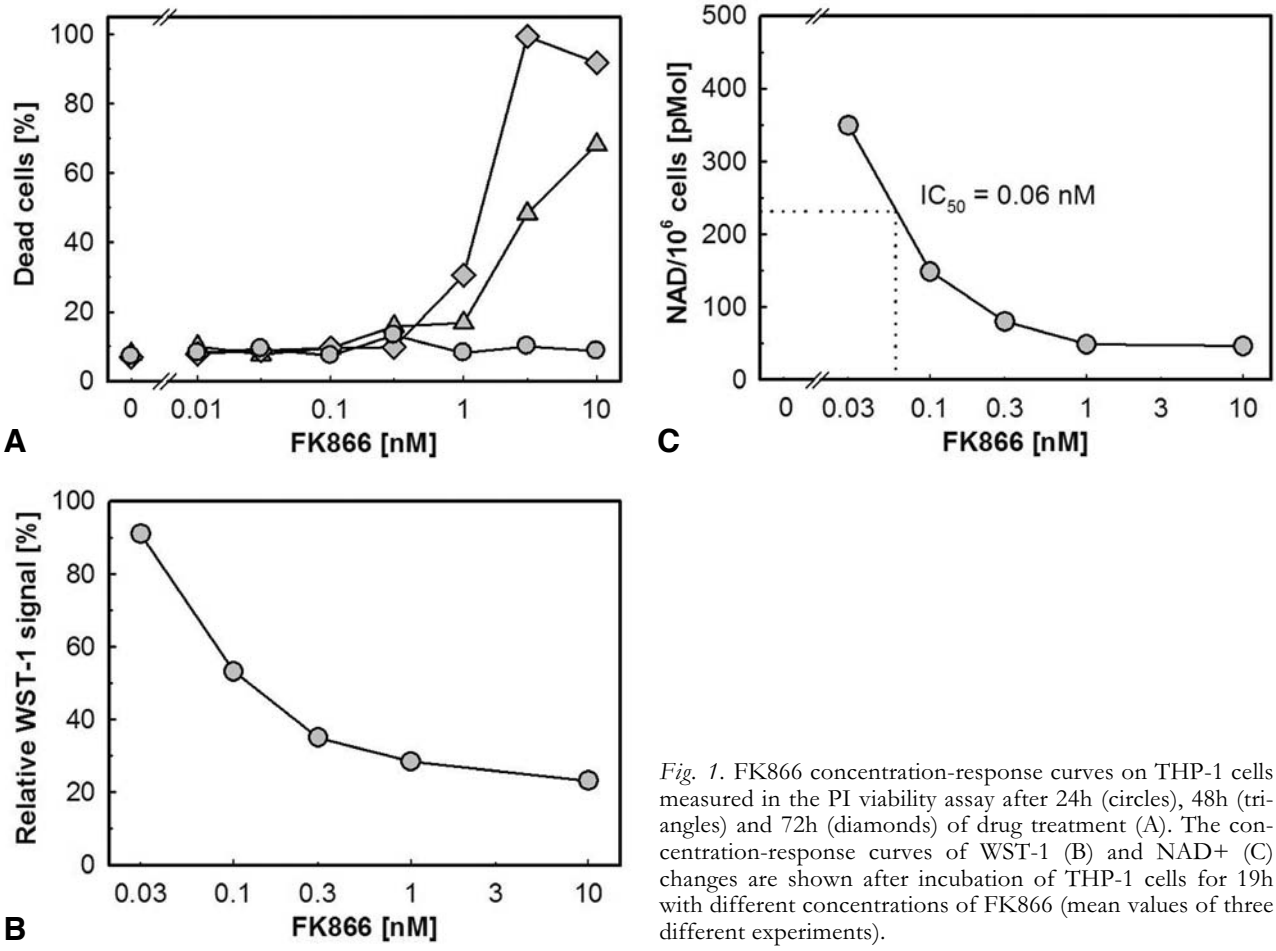


Fig. 1. FK866 concentration-response curves on THP-1 cells measured in the PI viability assay after 24h (circles), 48h (triangles) and 72h (diamonds) of drug treatment (A). The concentration-response curves of WST-1 (B) and NAD+ (C) changes are shown after incubation of THP-1 cells for 19h with different concentrations of FK866 (mean values of three different experiments).

taneously with daunorubicin and the PI assay was performed 24h, 48h and 72h after starting treatment. After 24 h treatment we did not see any differences between the two treatment modalities and less than half of the cells treated with daunorubicin alone or in combination with FK866 became PI-permeable (data not shown). Nevertheless we demonstrated that co-incubation with FK866 resulted in chemosensitization of THP-1 cells to cytotoxic effects of daunorubicin: The IC_{50} for daunorubicin was lower after 48 h simultaneous exposure to FK866, as compared to the cells that were treated with daunorubicin alone (6.5×10^{-8} M versus 2.3×10^{-7} M, Fig. 2A). However, the potentiating effect of co-incubation with FK866 on daunorubicin-induced cell death in THP-1 cells was transient and we could not observe any influence of simultaneous addition of FK866 on the IC_{50} for daunorubicin after 72h treatment (Fig. 2B).

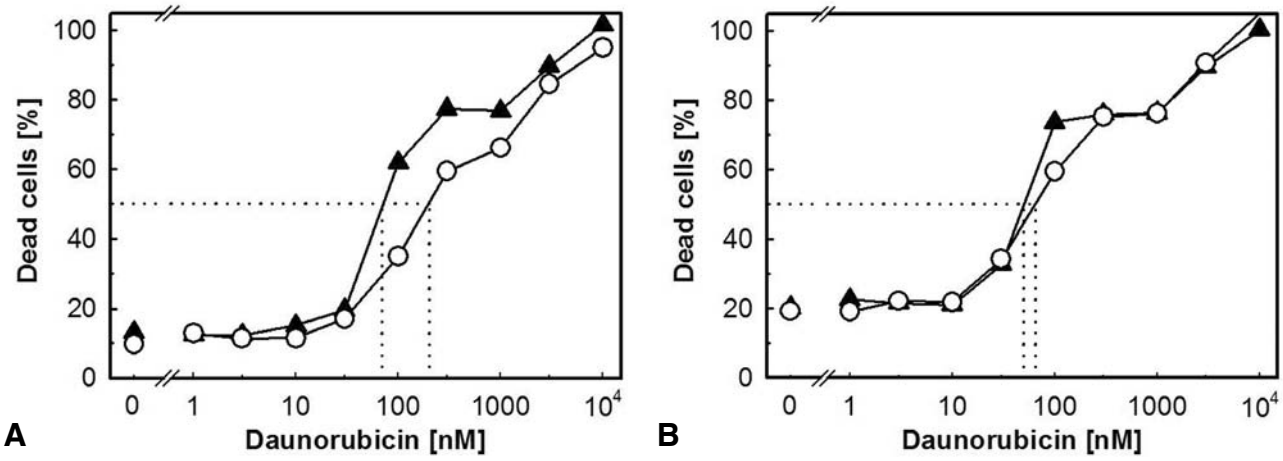


Fig. 2. Concentration-response curves on THP-1 cells for daunorubicin added alone (open circles) or in combination with FK866 (filled triangles) as measured in the PI assay after 48h (A) and 72h (B) treatment, respectively. Mean values of quadruplicate samples in at least three separate experiments are shown.

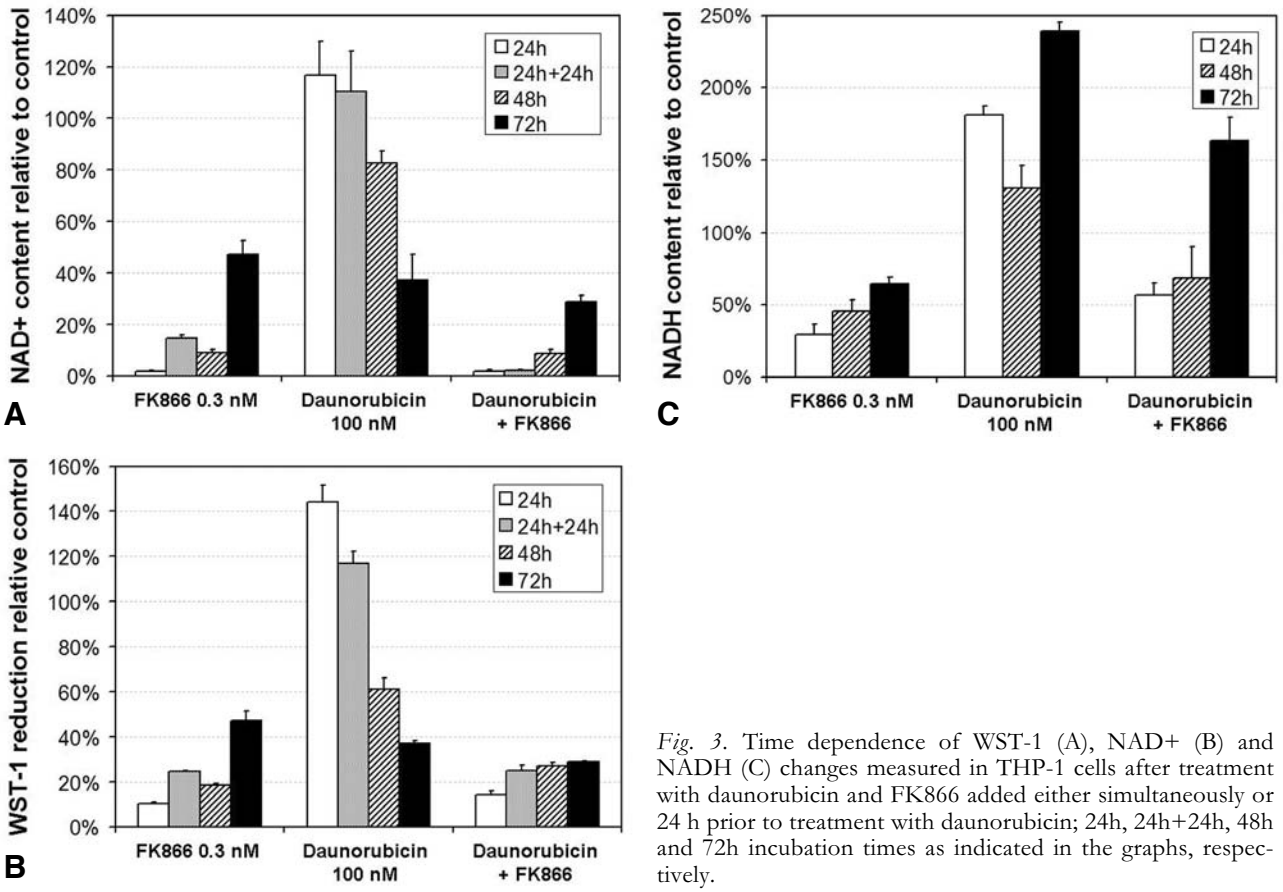


Fig. 3. Time dependence of WST-1 (A), NAD+ (B) and NADH (C) changes measured in THP-1 cells after treatment with daunorubicin and FK866 added either simultaneously or 24 h prior to treatment with daunorubicin; 24h, 24h+24h, 48h and 72h incubation times as indicated in the graphs, respectively.

Further we examined the effects of 10^{-7} M daunorubicin on the intracellular NADPH content and on the function of NAD-dependent dehydrogenases alone or in combination with 3×10^{-10} M FK866. FK866 was either added simultaneously or 24 hours before beginning the incubation with daunorubicin. Surprisingly, the intracellular content of pyridine nucleotides and the WST-1 reduction capacity were increased after 24 hours incubation with daunorubicin. After 3 days of treatment a drop in cellular reduction capacity and intracellular NAD^+ content to less than half of the control levels could be observed, whereas NADH (Fig. 3C) and NADPH (not shown) remained still elevated. On the contrary, FK866, added alone or in combination with daunorubicin, efficiently depleted intracellular NAD^+ pools and decreased WST-1-reduction by over 90% at 24 hours after starting treatment. Some recovery to nearly 50% of control levels was seen after 72 hours (Fig. 3 A, B). Intracellular NADH and NADPH levels remained also decreased (not shown).

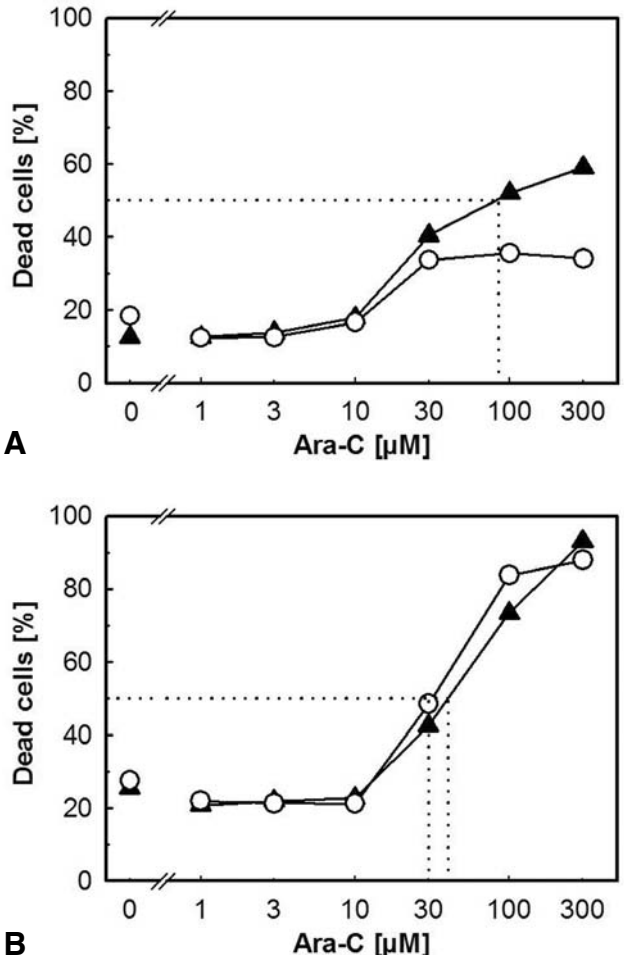
Effect of FK866 on Cell Death Induced by Ara-C in THP-1 Cells

Combination experiments with Ara-C were conducted by simultaneous addition of 3×10^{-10} M FK866 and different concentrations of Ara-C to THP-1 cells. Cell viability was estimated 48, 72, and 96 hours after starting treatment using the PI test. At 48 hours we could not see any advantage of the FK866/Ara-C combination compared to the treatment of THP-1 cells with Ara-C alone (not shown). Pretreatment with FK866 slightly potentiated cytotoxicity of Ara-C: after 72 hours treatment the IC_{50} for Ara-C in combination with FK866 was 8×10^{-5} M, whereas only 35% of cells died after exposure to the same concentration of Ara-C without FK866 (Fig. 4A). Similar to results obtained in combination experiments with daunorubicin, the chemosensitizing effect of FK866 for Ara-C-induced cell death was transient: we could not observe any significant differences in cell survival after 96 h of co-incubation with FK866 (Fig. 4B).

In contrast to daunorubicin, exposure to 10^{-4} M Ara-C decreased intracellular NAD^+, NADH and WST-1-reduction levels in THP-1 cells by more than 70% after 48 hours treatment. The extent of NAD^+ and NADH depletion, and of the decrease in WST-1 reduction that was observed after 24 and 48 hours combination treatment using 3×10^{-10} M FK866 and Ara-C, was similar as compared to the incubation with FK866 alone (Fig. 4C).

Chemosensitizing Effects of FK866 for MNNG-induced Cytotoxicity on THP-1 and K562 Cells

MNNG is an alkylating agent that induces DNA single strand breaks and is therefore a strong activator of PARP [16]. Because PARP utilises NAD as a substrate



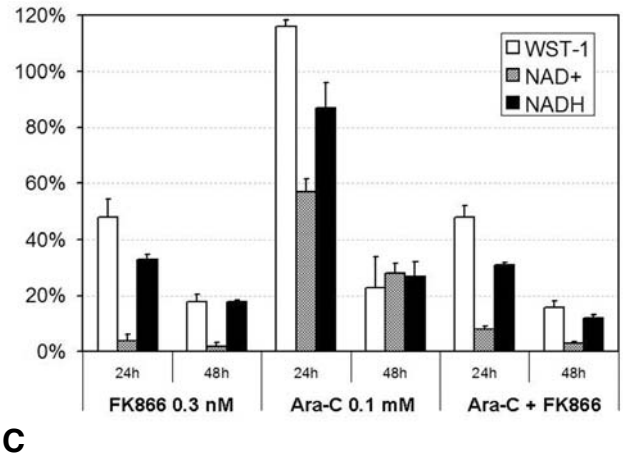


Fig. 4. Concentration-response curves on THP-1 cells for Ara-C added alone (open circles) or in combination with FK866 (filled triangles) as measured in the PI assay; 72h (A) and 96h (B). Time dependence of WST-1, NAD+ and NADH changes after treatment with FK866, Ara-C and the combination of both drugs; 24h and 48h drug incubation (C).

for poly-adenylation of proteins, we investigated the effects of MNNG on cells depleted of NAD by FK866 treatment. Prior to treatment with MNNG, the cells were pre-incubated with four different concentrations of FK866 for 24 hours. Cell viability was tested using the PI assay 24 hours after beginning the treatment with MNNG. Pre-incubation of THP-1 cells with 10^{-10} M FK866 did not significantly potentiate MNNG-induced cytotoxicity. However, when higher concentrations of FK866 (3×10^{-10} M or 10^{-9} M) were used, we observed that the concentration-response curve was shifted to lower drug concentrations indicating a decrease of EC_{50} for MNNG: EC_{50} values were calculated at 9×10^{-7} g/ml, and 2×10^{-7} g/ml, respectively, while EC_{50} for MNNG alone was 1.8×10^{-6} g/ml (Fig. 5A). We observed some intrinsic cytotoxicity of FK866 only at a concentration of 3×10^{-9} M: more than 30% of cells became permeable for PI (not shown). Pre-incubation of THP-1 cells with 3×10^{-10} M FK866 for 16 hours also promoted cell death in MNNG-exposed cells (Fig. 7A). However, when we reduced the pre-treatment time with FK866 to 8 hours, the synergistic effect of FK866 on MNNG-induced cell death in THP-1 cells could still be observed, but was less pronounced (Fig. 5B). The sensitizing effect of FK866 on MNNG-induced cytotoxicity was also demonstrated in the K562 cell line: when cells were pre-incubated with 10^{-8} M FK866 for 16 hours, EC_{50} for MNNG was decreased from 7×10^{-6} g/ml to 6×10^{-7} g/ml 36 hours after commencing treatment (Fig. 5C).

Alteration of the Intracellular NAD^+ Content and WST-1 Reduction after Treatment of THP-1 Cells with MNNG alone or in Combination with FK866

4 hours after begin of treatment with 1μg/ml MNNG we observed a decrease both of WST-1 reduction capacity and of intracellular NAD^+ content in THP-1 cells to nearly 50% of control levels. When THP-1 cells were pre-incubated with two different concentrations of FK866 for 24 hours, the alterations in WST-1 reduction became even more pronounced: using 10^{-10} M FK866 we achieved a decline of WST-1 reduction below 30% of controls, after pre-incubation with 10^{-9} M FK866 WST-1 reduction capacity dropped to under 5% of control levels, the difference was statistically significant ($p < 0.05$; Fig. 6A). In the cells pre-treated with 10^{-10} M FK866 followed by MNNG treatment, the intracellular NAD^+ levels remained unaltered, whereas a drop of the NAD^+ content was observed already after 0.5 hours treatment with MNNG in the cells that had been pre-incubated with 10^{-9} M FK866. Intracellular NAD^+ levels remained barely detectable 4 hours after starting treatment with this concentration of FK866 ($p < 0.05$; Fig. 6B).

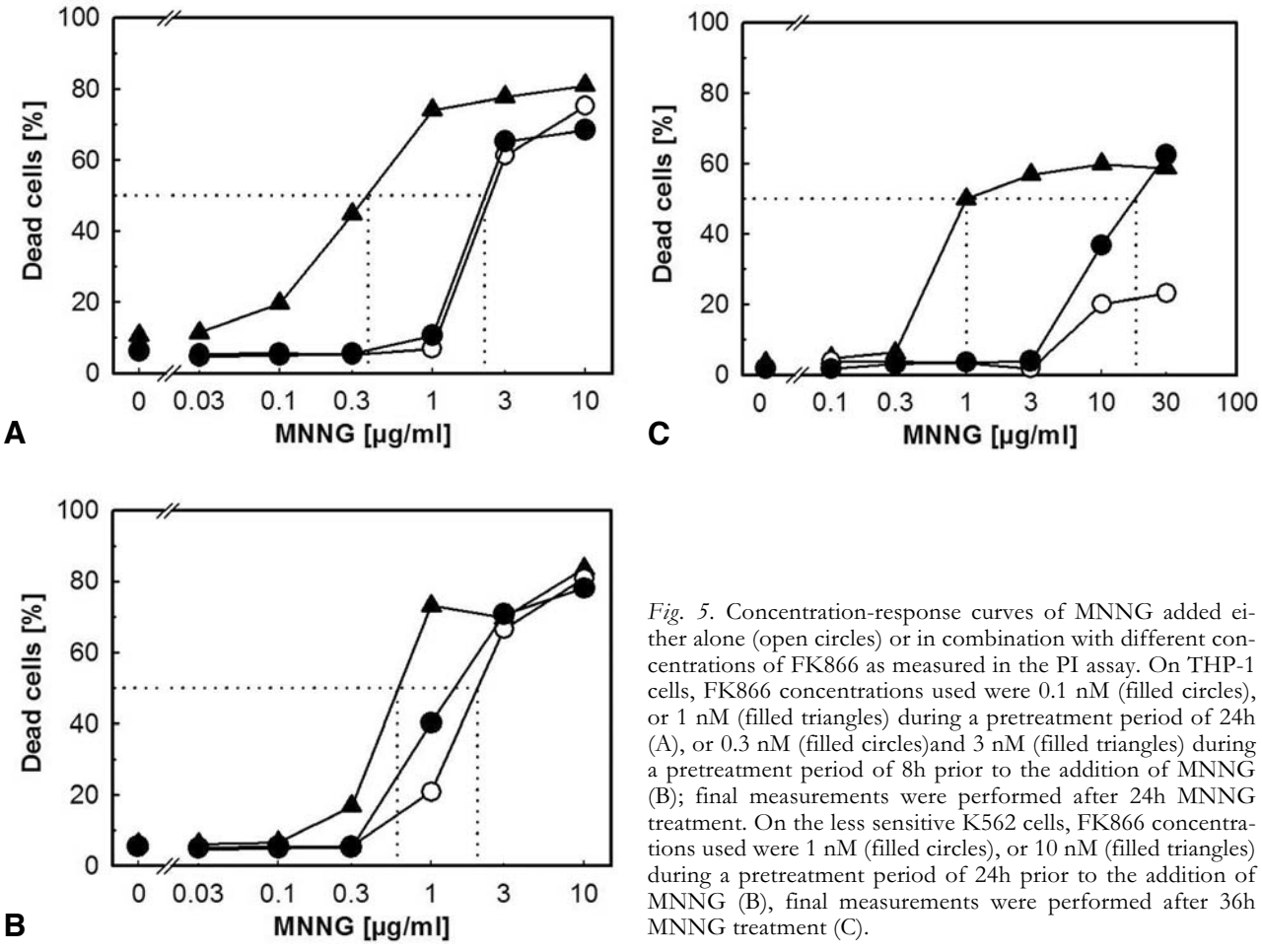


Fig. 5. Concentration-response curves of MNNG added either alone (open circles) or in combination with different concentrations of FK866 as measured in the PI assay. On THP-1 cells, FK866 concentrations used were 0.1 nM (filled circles), or 1 nM (filled triangles) during a pretreatment period of 24h (A), or 0.3 nM (filled circles)and 3 nM (filled triangles) during a pretreatment period of 8h prior to the addition of MNNG (B); final measurements were performed after 24h MNNG treatment. On the less sensitive K562 cells, FK866 concentrations used were 1 nM (filled circles), or 10 nM (filled triangles) during a pretreatment period of 24h prior to the addition of MNNG (B), final measurements were performed after 36h MNNG treatment (C).

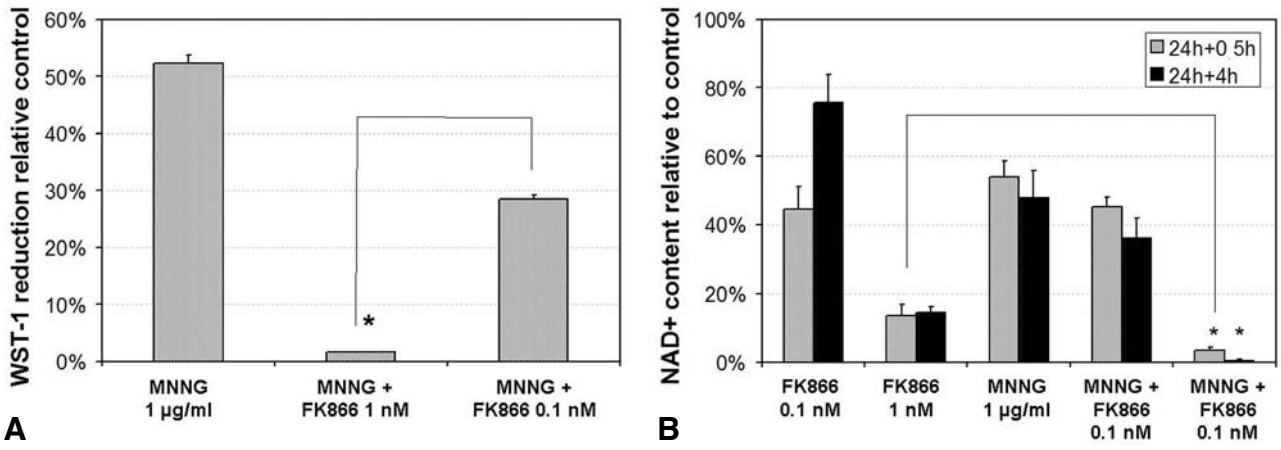


Fig. 6. Changes in WST-1 reduction (A) and of intracellular NAD+ contents (B) of THP-1 cells after preincubation with different concentrations of FK866 for 24h and subsequent exposure to MNNG for 0.5 and 4 hours. Asterisks indicate a statistically significant decline of WST-1 reduction as well as NAD+-contents in cells pretreated with 1 nM FK866 compared with lower drug concentrations (0.1 nM).

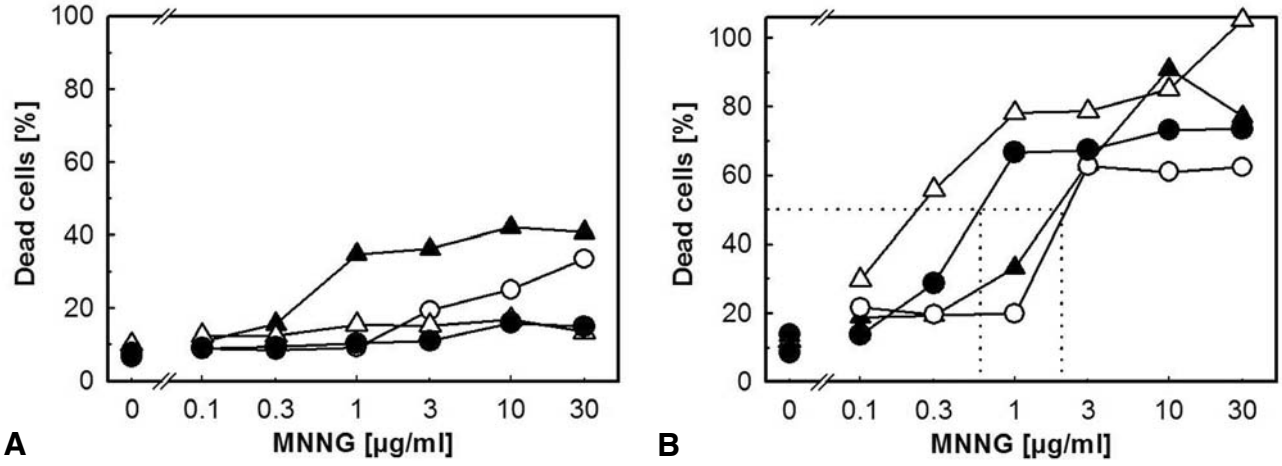


Fig. 7. Effect of the PARP-inhibitor 3-aminobenzamide on the concentration-response curves on THP-1 cells for MNNG added alone (MNNG, open circles; MNNG+3-AB, filled circles) or in combination with 0.1 nM FK866 (FK866+MNNG, filled triangles; FK866+MNNG+3-AB, open triangles); FK866 was added 16h prior to starting treatment with MNNG ; cell viability was measured after 19h (A) and 72h (B) combination treatment, respectively, using the PI assay.

Protective Effect of the PARP Inhibitor 3-Aminobenzamide on Cell Death Induced by MNNG alone or in Combination with FK866

We showed that pre-incubation with 3 mM of the PARP inhibitor 3-aminobenzamide markedly decreased the proportion of cells that became PI-positive after treatment with MNNG for 19 hours. The protective effect of 3-aminobenzamide could also be observed in cells that were pre-incubated with FK866 (Fig. 7A). The WST-1 assay, when conducted 19 hours after starting treatment with MNNG, revealed a drop of cellular reduction capacity only in the cells that were pre-treated with FK866 (not shown). 72 hours after starting treatment with MNNG we could not demonstrate any protective impact of 3-aminobenzamide on MNNG-treated cells. On the contrary, an increased proportion of cells that underwent cell death was observed in the cultures that were pre-treated both with FK866 and 3-aminobenzamide, as compared to the pre-treatment with FK866 alone (Fig. 7B).

Melphalan-induced Cytotoxicity on THP-1 and K562 Cells alone and after Pre-incubation with FK866

We further addressed the question whether the chemopotentiating effects of FK866 demonstrated on MNNG-induced cell death could also be observed in combination with other alkylating agents. Therefore, we tested the cytotoxicity on THP-1 and K562 cells after 48 hours exposure to Melphalan without or with pre-treatment with FK866 for 16 hours. The cytotoxic effects of Melphalan in both cell lines were only slightly more pronounced in the cells pre-incubated with FK866, particularly when higher concentrations of FK866 (10^{-9} M for THP-1 cells and 10^{-8} M for K562,

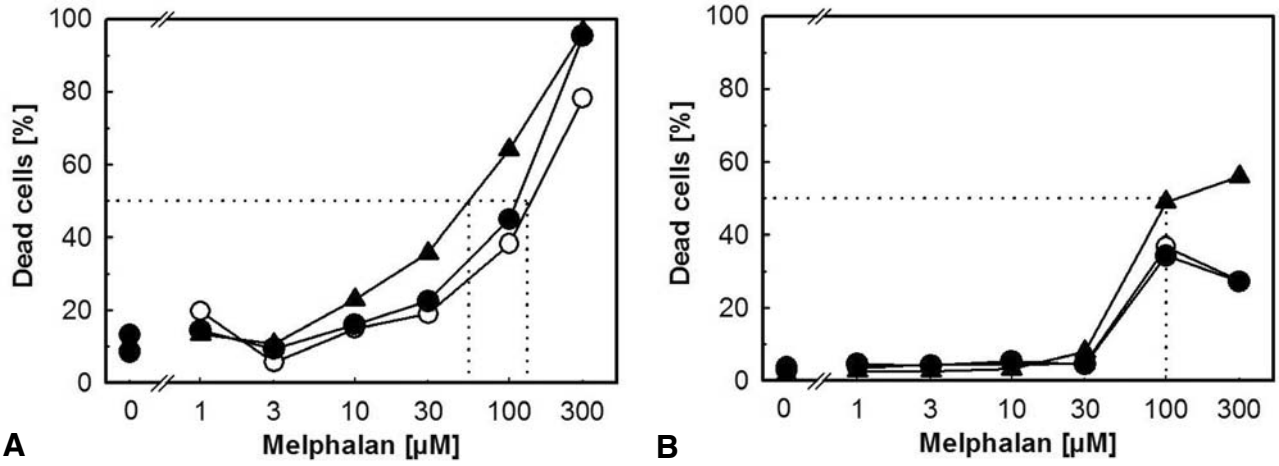


Fig. 8. Concentration-response curves on THP-1 cells (A) and K562 cells (B) for Melphalan added alone (open circles) or in combination with FK866 (0.3 nM, filled circles; or 1 nM, filled triangles) as measured in the PI assay; 48h combination treatment.

respectively) were used. In THP-1 cells the IC_{50} for Melphalan alone after 48 hours was 1.5×10^{-4} g/ml compared with 5×10^{-5} g/ml for combination treatment with Melphalan and FK866 at the same time point. Contrary to MNNG-induced cell death, a shift of the concentration-response curve to the left in the cells pre-incubated with FK866 could not be observed (Fig. 8A). Similar effects were obtained in K562 cells 48 hours after starting treatment with Melphalan (Fig. 8B). When 10^{-4}M Melphalan was used together with 10^{-8}M FK866, 50% of K562 cells were dead, whereas only 35% of K562 cells became PI-permeable after treatment with 10^{-4} Melphalan alone.

Discussion

In the present study we showed that FK866 at concentrations which were neither cytotoxic nor cell growth inhibitory could decrease both intracellular NAD^+ content and WST-1 reduction capacity by more than 65%, and – to a lesser extent – of NADH levels. These effects were recorded already after 19 hours of treatment. The NAD depleting features of FK866 were by far superior as compared to the NAD analogs tiazofurin and selenazofurin [10]. Previously, those substances had been demonstrated to exert suppressive effects on the growth rate of haematopoietic cells with a concomitant NAD^+ decrease by only 25% [11]. The more efficient depletion of NAD by FK866 can be explained by its direct inhibition of the enzyme NAPRT which is involved in the nicotinamide pathway of NAD synthesis, whereas tiazofurin and selenazofurin act indirectly, being metabolized to form NAD analogs which in turn exert a feedback inhibition of NAD synthesis.

Further we observed that pre-treatment with FK866 did not increase the relative number of dead cells after treatment with daunorubicin and Ara-C, but only accelerated the onset of cell death in THP-1 cells. The possible reason for the absence of chemosensitization of cells against these drugs by FK866 may be the fact that daunorubicin and Ara-C did neither show a synergistic effect on the decrease of intracellular NAD^+ and NADH levels, nor reduce metabolic activity as indicated by the WST-1 assay. The influence of daunorubicin on the intracellular pool of pyridine nucleotides was antagonistic to that of FK866: both NAD^+ content and WST-1 reduction in THP-1 cells were higher as compared to control cells shortly after the beginning of treatment and gradually decreased with prolonged incubation time. Intracellular NADH and NADPH levels were even increased during treatment with daunorubicin, corresponding with a so-called "reductive stress" [12]. Although co-incubation with FK866 depleted NAD^+ levels, the daunorubicin mediated increase of NADH and NADPH levels could not be completely prevented by FK866. In contrast to daunorubicin, treatment with Ara-C decreased NAD^+ levels in THP-1 cells, but the NAD^+ depletion caused by FK866 was not further augmented by Ara-C. This result may explain why the combination of both drugs could only slightly and transiently improve cytotoxicity compared to treatment with Ara-C alone.

Our interest primarily focused on potential partners for combination therapy with the novel antitumour compound FK866. Cytotoxic chemotherapy with compounds that decrease intracellular NAD^+ levels even when used as single agents was considered more likely to be potentiated by pre-incubation and/or concomitant treatment with FK866. MNNG is a DNA damaging agent that causes extensive primary DNA single-strand breaks immediately after starting treatment. Subsequent PARP activation in an attempt to activate DNA repair leads to rapid NAD^+ depletion which in turn may account for significant cytotoxicity of MNNG [13]. In support of this view, treatment with the PARP inhibitor 3-aminobenzamide delays cell death evoked by strong DNA damaging substances [13]. In our experiments, contrary to daunorubicin- or Ara-C-induced cell death, more than a half of the cells died already 24 hours after starting incubation with MNNG. Then we added FK866 8-24 hours before treatment with MNNG so that the intracellular NAD^+ levels were already depleted before PARP was activated by DNA damage. Pre-incubation with FK866 at concentrations above 10^{-10} M could clearly sensitize both THP-1 and K562 cells to the cytolytic effects of

MNNG. Cell death was even observed in FK866 pretreated cells using non-toxic concentrations of MNNG.

In order to further substantiate the hypothesis that the chemo-potentiating effects of FK866 on MNNG-induced cell death are indeed due to decreased NAD^+ levels, we measured the intracellular NAD^+ content and WST-1 reduction capacity of THP-1 cells after exposure to MNNG. Although treatment with both 10^{-9} M and 10^{-10} M FK866 decreased WST-1 reduction and the intracellular NAD^+ content 4 hours after starting treatment, the extent of the decrease was much stronger when higher concentrations were used (Fig. 6A). Only 10^{-9} M FK866 showed a synergistic effect when applied together with a non-toxic concentration of MNNG: a drop in WST-1 reduction and intracellular NAD^+ levels to below 10% of control levels was observed. Apparently, only a massive (> 80%) decrease of intracellular NAD^+ content can sensitize cells to the cytotoxic effects of MNNG.

We also demonstrated that PARP activation plays a key role during MNNG-induced cell death: inhibition of PARP by 3-aminobenzamide delayed cell death induced by MNNG also in FK866 pretreated cells. However, the PI-exclusion assay conducted at a later time point (72 hours) revealed that even more cells were dead when preincubated with both 3-aminobenzamide and FK866, as compared to the cells pretreated with FK866 alone. Since PARP requires NAD^+ for its function and it is well known that PARP inhibitors potentiate the cytotoxicity of various DNA-damaging agents [14, 15], we hypothesize that NAD^+ shortage after preincubation with FK866 compromised the proper function of PARP. As a consequence, MNNG-induced DNA strand breaks could not be repaired and the cells died. The observation that the effect of pretreatment with FK866 was less pronounced on Melphalan-induced cell death in THP-1 and K562 cells may be explained by the considerably protracted kinetics of cell death after treatment with Melphalan as compared to MNNG. Unlike MNNG, Melphalan does not primarily activate PARP but triggers apoptosis at a much slower rate by NAD- and ATP-depletion [16].

In conclusion, the present data show that FK866 may be particularly useful to enhance the antitumor activity of conventional chemotherapeutic drugs that have an intrinsic NAD^+-depleting effect. These results suggest clinical studies of FK866 in combination with DNA-damaging agents like MNNG.

Note: **FK866** has been licensed by **Apoxis S.A., Lausanne, Switzerland,** and is now in clinical development under the name of **APO866**.

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Received: January 17, 2006 / Accepted: May 12, 2006

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