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Radioprotective Effects of Flurbiprofen and Its Nitroderivative

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Summary

Radioprotective effects of two non-steroidal anti-inflammatory drugs, flurbiprofen (FBP) and its novel nitroderivative flurbiprofen 4-nitroxybutylester (NO-FBP), which exhibits decreased gastrointestinal toxicity, were compared in mice. The drugs were administered in equimolar single doses, 2 hours before whole-body gamma-irradiation of the animals. After a sublethal radiation dose of 6.5 Gy, significantly increased numbers of endogenous haemopoietic spleen colonies and enhanced granulopoiesis were found in mice given either FBP or NO-FBP, when compared to vehicle-treated controls. There were no differences in the effectiveness of either drug to enhance postirradiation haemopoietic recovery. Survival of FBP- or NO-FBP-treated mice subjected to a lethal dose of 9.5 Gy was slightly but insignificantly enhanced, both drugs showing the same effect. These results clearly indicate the ability of both drugs to enhance haemopoietic recovery after sublethal radiation exposure and the absence of unfavourable effects under higher radiation doses. Because of its lower potential for gastrointestinal damage, NO-FBP seems to be a promising drug, which can find a use in the protection of postirradiation myelosuppression.

Key words
Inhibitors of prostaglandin production – Flurbiprofen nitroderivative – Radioprotection – Haemopoiesis

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely employed in clinical practice for the treatment of a number of pathological conditions. The beneficial effects of these drugs can be attributed to their ability to suppress the synthesis of prostaglandins (PGs) (Flower 1974). Since PGs of the E type play a role of negative feedback factors in the regulation of granulopoiesis and probably also in the proliferation of more primitive haemopoietic cells (Kurland and Moore 1977, Fontagné 1980, Pelus 1989), it seems to be possible to use the NSAID-mediated suppression of their generation to modulate myelopoiesis positively. Indeed, the treatment of mice with inhibitors of prostaglandin synthesis, such as indomethacin or diclofenac, was found to enhance the recovery of haemopoiesis after sublethal irradiation (Pospíšil *et al.* 1986, 1989, Nishiguchi *et al.* 1990, Kozubík *et al.* 1989, 1994, Hofer *et al.* 1996). In addition, NSAIDs have been found to act synergistically with immunomodulators (Pospíšil *et al.* 1992, Hofer *et al.* 1993, Fedoročko and Macková 1996) and sulfhydryl compounds (Kozubík *et al.* 1990) to protect haemopoiesis in irradiated mice.

However, the use of NSAIDs can be associated with significant adverse gastrointestinal effects, e.g. ulceration, bleeding, perforation and increase of enterocytic permeability (Wang *et al.* 1989, Bjarnason *et al.* 1991). These effects are also directly linked to the ability of NSAIDs to inhibit the synthesis of prostaglandins which have cytoprotective activity in the gastrointestinal mucosa (Mahmud *et al.* 1996).

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The adverse gastrointestinal effects can limit the usefulness of NSAIDs particularly under situations of impaired gastrointestinal functions, including the radiation syndrome. The survival of mice subjected to a lethal dose and treated with a repeated postirradiation administration of indomethacin or diclofenac was decreased (Hofer *et al.* 1992). Similarly, reduced survival and appearance of early deaths were seen in mice treated with single high indomethacin dose before irradiation (Floersheim 1994). These effects can be attributed to enhanced postirradiation intestinal damage.

A number of new derivatives of common NSAIDs have been recently synthesized with the aim to obtain a drug with reduced gastrointestinal side effects. Among them, a nitroderivative of the commonly clinically used NSAID flurbiprofen (FBP), flurbiprofen 4-nitroxybutylester (NO-FBP), has been recently developed and tested in experimental animals (Wallace *et al.* 1994, 1995). In NO-FBP, the nitroxybutylester group releases nitric oxide in tissues of the gastrointestinal tract. Nitric oxide is known as a protector of the gastrointestinal mucosa (MacNaughton *et al.* 1989, Wallace 1996). Investigation of the effects of FBP and NO-FBP indicated comparable suppression of prostaglandin synthesis and anti-inflammatory action, together with a highly decreased incidence of the adverse gastrointestinal effects of the nitroderivative (Wallace *et al.* 1994).

In the experiments presented here, FBP and NO-FBP were tested as potential radioprotective agents. The effects of the drugs on stimulation of haemopoiesis after sublethal irradiation and on the mortality of experimental animals irradiated with a lethal dose were compared.

Material and Methods

Experimental animals

Male (CBAxC57Bl/10) F1 mice, aged 3 months and with average weight of 25 g at the beginning of the experiment, were used throughout this study. The animals (20 individuals per cage) were kept under controlled illumination (LD 12:12) at a temperature of 22±1 °C. A pelleted sterilized diet (DOS 2ST Velaz) and HCl-treated tap water (pH 2–3) were given *ad libitum*. Both control and experimental procedures were carried out in the group of mice from the same cage.

Irradiation

The mice were irradiated with single whole-body doses using a ^{60}Co gamma-ray source (Chisostat, Chirana) at a dose rate of 0.3 Gy/min. The mice were placed individually in perforated plexiglass chambers in a slowly rotating circular container during the irradiation.

Administration of drugs

Flurbiprofen ([±]-2-fluoro-alpha-methyl-4-biphenyl-acetic acid) (Sigma, St. Louis, MO) was dissolved in undiluted dimethylsulfoxide (DMSO) and administered in a dose of 0.3 mg per mouse, i.e. about 10 mg/kg. Such a dose of FBP was found to exert a marked anti-inflammatory effect when tested on the carrageenan-induced paw oedema (Wallace *et al.* 1994). Flurbiprofen 4-nitroxybutylester (kindly provided by Dr. P. Del Soldato of Pharmaceutical Discovery Service, Milan, Italy) was also dissolved in undiluted DMSO and administered in a dose equimolar to that of flurbiprofen, i.e. 0.47 mg per mouse. Both drugs were injected intraperitoneally in volumes of 0.05 ml 2 hours before irradiation. Control animals received the same volumes of DMSO prior to gamma-irradiation. DMSO was used as a solvent because of the very poor solubility of NO-FBP in saline. It was found in preliminary experiments that the dose of 0.05 ml of undiluted DMSO did not influence the parameters evaluated under the used experimental conditions (results not shown).

Haematological methods

In experiments evaluating haemopoiesis, the material was sampled on days 7, 10, 13 and 16 after sublethal irradiation. Complete haemopoietic examinations were performed on all days of sampling. Blood samples were taken from the tail vein. Mice were killed by cervical dislocation, their femora were dissected, and marrow cells were flushed from the bone. Blood cell counts and numbers of nucleated cells of the bone marrow were determined using a Coulter Counter, and differential counts were performed using smear preparations stained with the May-Grünwald-Giemsa method. Endogenous spleen colony-forming units (CFU-S) (Till and McCulloch 1963) were counted on day 10 after irradiation. The spleens were removed and fixed in Bouin's solution. Macroscopically visible nodules larger than 0.4 mm in diameter were counted. In addition, the spleens were embedded in paraffin and used for histological analysis. A single midline longitudinal section was considered to be a representative sample of each spleen (Inoue *et al.* 1984) and was used for counting histologically evaluable colonies (more than 10 cells were considered to be a colony) and for counting granulopoietic clusters (either separate granulopoietic colonies or distinguishable granulopoietic parts in mixed colonies). The parameter of granulopoietic clusters was used as an approximate indicator of splenic granulopoiesis.

Survival

In experiments evaluating the mortality of animals, deaths were recorded in daily intervals for 30 days after lethal irradiation.

Statistical analysis

The values given in the tables and figures represent the mean ± S.E.M. For each point, 6 to 17 animals were used. Statistical significance of the results of experiments testing haemopoietic recovery in sublethally irradiated mice was evaluated using One-Way Analysis of Variance or the Kruskal-Wallis One-Way Analysis by Ranks. Survival data were analysed by life-table method using the "long-rank" test (Peto *et al.* 1977). The significance level was set at $P<0.05$.

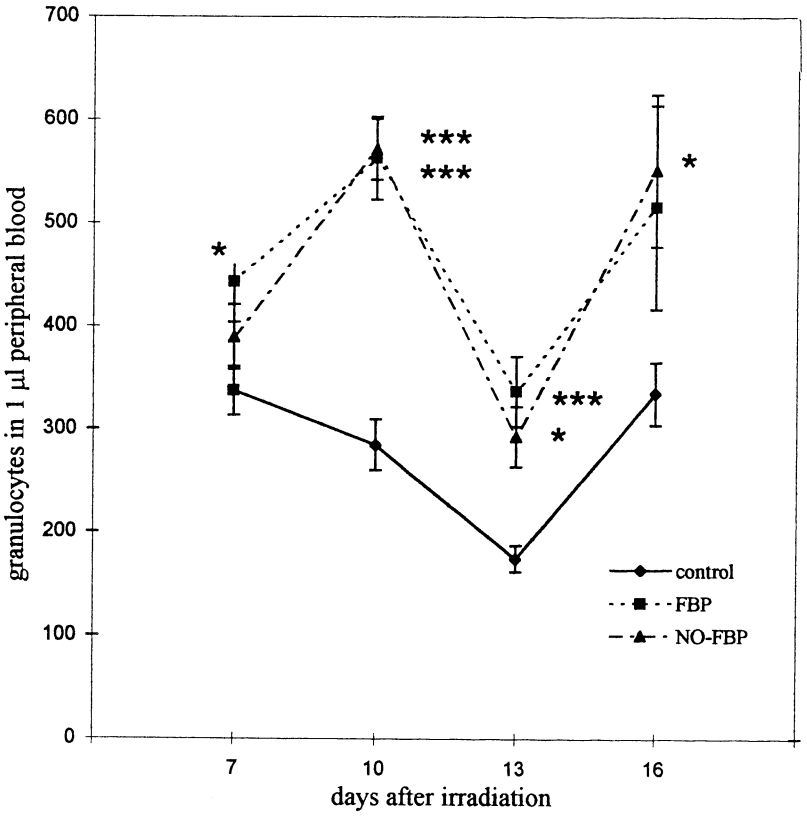

Fig. 1. *Number of granulocytes in 1 µl of peripheral blood on days 7, 10, 13 and 16 after irradiation with 6.5 Gy in control mice (Control), mice pretreated with flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP), 12 to 14 mice per group were used. Statistical significance as compared to controls: * $P<0.05$ and *** $P<0.001$.*

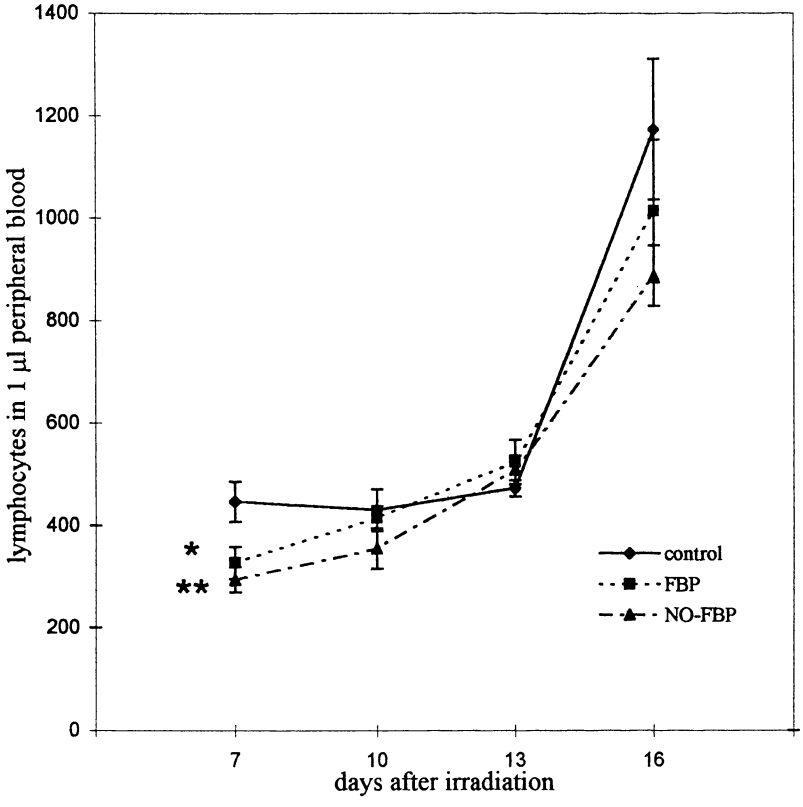

Fig. 2. *Numbers of lymphocytes in 1 µl of peripheral blood on days 7, 10, 13 and 16 after irradiation with 6.5 Gy in control mice (Control), mice pretreated with flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP), 12 to 14 mice per group were used. Statistical significance as compared to controls: * $P<0.05$ and ** $P<0.01$.*

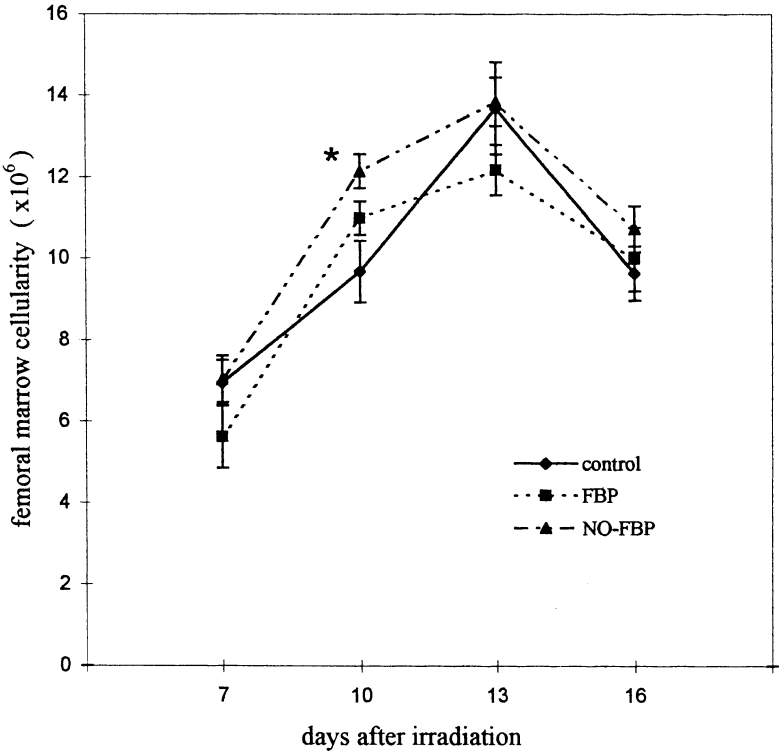

Fig. 3. *Femoral marrow cellularity on days 7, 10, 13 and 16 after irradiation with 6.5 Gy in control mice (Control), mice pretreated with flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP), 12 to 14 mice per group were used. Statistical significance as compared to controls: * P<0.05.*

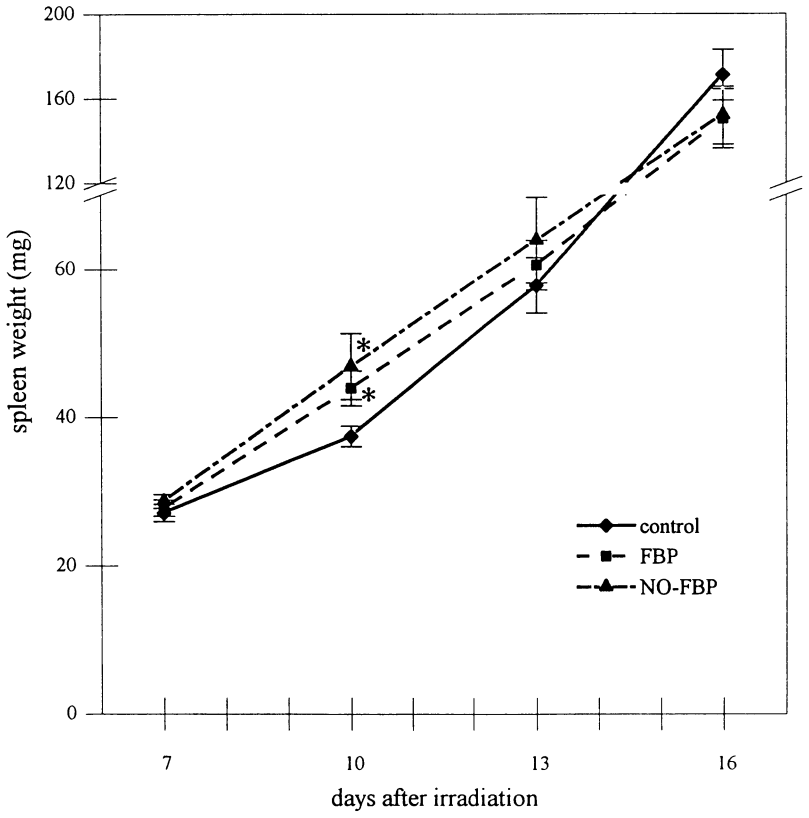

Fig. 4. *Spleen weight on days 7, 10, 13 and 16 after irradiation with 6.5 Gy in control mice (Control), mice pretreated with flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP), 12 to 14 mice per group were used. Statistical significance as compared to controls: * P<0.05.*

Results

Differential counting of white blood cells in the blood of mice irradiated with a sublethal dose of 6.5 Gy revealed effects of the administration of FBP or NO-FBP preferentially on granulocytes. In comparison with the controls, granulocyte numbers were found to be significantly higher on days 7, 10 and 13 in FBP-

treated mice and on days 10, 13 and 16 in NO-FBP-treated mice (Fig. 1). There were no significant differences in granulocyte numbers between animals administered FBP and NO-FBP at any time interval studied. No significant differences in blood lymphocyte counts on days 10, 13 and 16 were found by any comparison between the three groups (Fig. 2). Only on day 7 after irradiation, the drug-treated groups exhibited lower values of blood lymphocytes as compared to the controls. This effect might be a consequence of the nonspecific stress action of the drugs. Blood erythrocyte counts were not influenced by the treatments employed (data not shown).

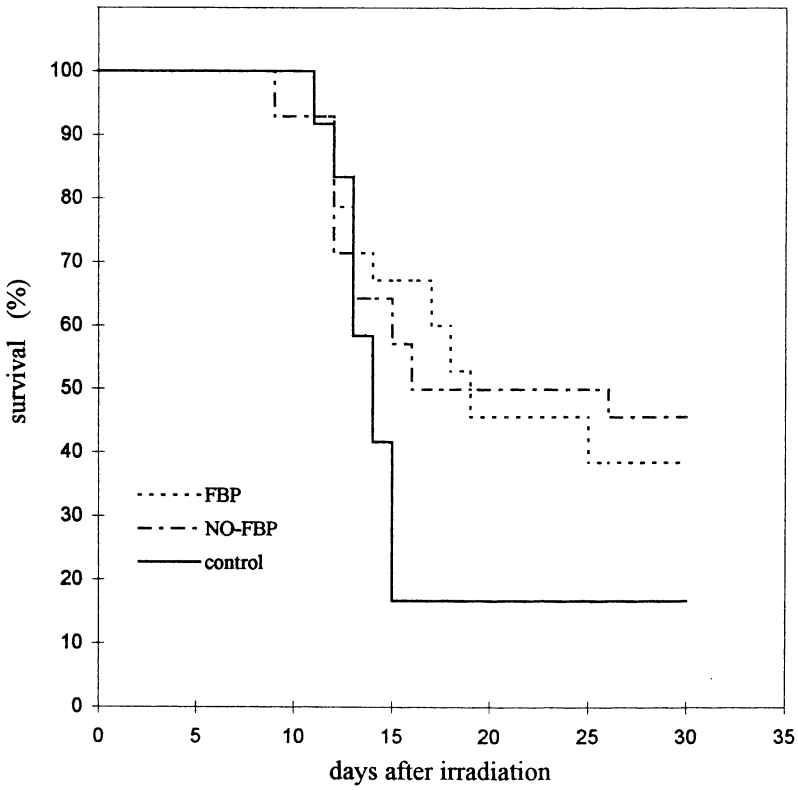

Fig. 5. *Survival of mice irradiated with 9.5 Gy, pretreated two hours before irradiation with solvent (Control), flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP). The respective groups comprised 12 to 14 animals, data were obtained from two replicate experiments. Differences in mortality rate between the three groups are not significant.*

Table 1. Indices of postirradiation haemopoiesis in mice pretreated with flurbiprofen (FBP) or flurbiprofen 4-nitroxybutylester (NO-FBP) and in control mice on day 10 after sublethal irradiation with 6.5 Gy

Haematological parameters assessed 10 days after irradiation	Controls	FBP	NO-FBP
Number of macroscopic spleen colonies	6.25±0.78	13.00±0.84***	16.00±1.19***
Number of microscopic spleen colonies	6.33±1.20	13.14±1.35*	15.42±2.06*
Spleen weight (mg)	37.43±1.42	43.93±2.35*	46.90±4.46*
Femoral bone marrow cellularity (x10^6)	9.67±0.76	10.98±0.41	12.14±0.42*
Number of granulocytic cells in bone marrow (x10^6)	3.27±0.27	5.15±0.29***	5.68±0.43***
Number of granulopoietic clusters in spleen	2.67±0.61	7.00±1.00*	9.57±1.25***

*Six to 17 mice per group were used. Statistical significance: * $P<0.05$ and *** $P<0.001$ as compared to the controls.*

The kinetics of femoral marrow cellularity and the spleen weight are shown in Fig. 3 and Fig. 4, respectively. The only statistically significant differences were found on day 10. For this reason, the spleen and the bone marrow from day 10 were subjected to a more detailed analysis. Table 1 summarizes the findings from day 10 in six indices. Animals given FBP or NO-FBP exhibited significantly

higher values of all parameters (numbers of macro- and microscopic spleen colonies and numbers of granulopoietic clusters, spleen weight, femoral marrow cellularity and numbers of granulocytic cells in femoral marrow), with the exception of the difference in marrow cellularity between FBP-treated and control mice. No significant differences between animals given FBP and NO-FBP were observed. The differences in the numbers of erythroid and lymphoid cells in femoral marrow between any of the groups were not significant (data not shown).

After irradiation with an almost absolute lethal dose of 9.5 Gy (Fig. 5), survival of the mice treated with FBP or NO-FBP was found to be slightly, but not significantly higher in comparison with the control group. No significant differences between FBP- and NO-FBP-treated animals were observed.

Discussion

The results presented here clearly suggest that equimolar doses of FBP and NO-FBP induce equal protective effects in terms of haemopoietic recovery in sublethally irradiated mice. Higher numbers of both macroscopic and microscopic spleen colonies observed after administration of FBP or NO-FBP provide evidence for increased proliferation at the level of haemopoietic stem cells. The investigations of peripheral blood and bone marrow smears as well as of granulopoietic clusters in the spleen showed that the positive effect of the tested drugs also comprises their ability to enhance the intensity of granulopoiesis. An increase in the proliferation of the stem cells in haemopoietic tissues, in differentiation towards the granulocyte lineage, and enhancement of maturation and release of granulocytes into blood circulation may reduce the severity of the bone marrow syndrome and enhance the resistance to infection.

The enhancement of the postirradiation haemopoietic recovery after FBP or NO-FBP pretreatment is comparable to that observed after administration of other NSAIDs such as indomethacin and diclofenac (Pospíšil *et al.* 1986, 1989, Nishigushi *et al.* 1990, Kozubík *et al.* 1989, 1994, Hofer *et al.* 1996). This finding corroborates the hypothesis of the similar mechanism of the haemopoiesis-stimulating action of the two drugs, i.e. the inhibition of prostaglandin synthesis. As was shown previously, the beneficial protective influence of NSAIDs is not due to any changes of radiosensitivity of haemopoietic cells, but is induced by an increase of their proliferative activity (Pospíšil *et al.* 1986, Kozubík *et al.* 1994). The removal of the inhibitory action of prostaglandins on the proliferation of haemopoietic cells (Kurland and Moore 1977, Fontagné *et al.* 1980, Pelus 1989) can account for the accelerated haemopoietic recovery.

Our results show a moderate but insignificant enhancement of mice survival after the dose of 9.5 Gy in both drug-treated groups in comparison with the controls and the absence of differences in survival between the two drug-treated groups. Assuming the different gastrointestinal toxicity of FBP and NO-FBP, the lack of significant differences in survival between the two drug-treated groups can be due to a relatively small role of the gastrointestinal radiation syndrome after the nearly absolute lethal dose. Concerning the lack of significant radioprotective efficacy of both drugs in terms of postirradiation survival, one has to take into account that the haemopoiesis-stimulating effects of the drugs are logically less effective in the situation of greater haemopoietic damage and thus at a lower number of surviving haemopoietic stem cells. Nevertheless, the results of the presented survival experiments clearly suggest the relative safety of the drugs even under conditions of higher radiation doses.

The evidence that NO-FBP, a novel NSAID with an improved gastrointestinal tolerance, exhibits radioprotective effects on haemopoiesis might have important practical implications. It was demonstrated that NSAIDs can enhance therapeutic gain when combined with tumour radiotherapy in mice (Furuta *et al.* 1988). As summarized by Michalowski (1994), anti-inflammatory drugs tend to attenuate a range of prodromal, acute and chronic effects of radiation even in man. Such treatment strategy might be of importance particularly when preventing normal tissue damage, including the haemopoietic one, in patients submitted to radiotherapy and those accidentally exposed to radiation. Under these conditions, the use of drugs with lower efficacy for gastrointestinal damage and thus with a more favourable therapeutic index is clearly preferable.

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