Genotoxicity testing of antiparasitic nitrofurans in the Drosophila wing somatic mutation and recombination test

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Nifurtimox and eight structurally related 5-nitrofuran compounds active against *Trypanosoma cruzi* were tested for genotoxicity in the wing somatic mutation and recombination test in *Drosophila melanogaster*. Nifurtimox, compound ada and compound 1B were clearly mutagenic and recombinogenic whereas the remaining six compounds were negative. In contrast to the situation in bacterial mutagenicity tests, nitroreductase activity is probably not decisive for the genotoxicity of these compounds in Drosophila. The three non-genotoxic nitrofurans with high antiparasitic activity are promising candidates for the replacement of nifurtimox. However, these compounds require further genotoxicity testing in eukaryotic assay systems for a final evaluation.

Introduction

5-Nitrofurans are a large group of synthetic nitro compounds used worldwide for various purposes, as human and veterinary drugs, food additives or preservatives. For this reason, many possible routes of human exposure to these chemicals exist (for a review, see Bryan, 1978). Nifurtimox [1-((5-nitrofurfurylidene)amino)-3-methylthiomorpholine-1,1-dioxide] is the most effective 5-nitrofuran for the treatment of Chagas' disease which affects 10^7 people in Central and South America and whose aetiological agent is *Trypanosoma cruzi*. The trypanocidal effect of nifurtimox (nfx) may implicate several mechanisms such as direct inhibition of biosynthetic reactions (DNA, RNA and protein synthesis), as well as breakage and degradation of macromolecules by oxygen radicals generated in the parasite. Nevertheless, the knowledge of the mode of action of this drug on *T.cruzi* is still incomplete (Goijman and Stoppani, 1985; Goijman *et al.*, 1985). In addition, nfx possesses some side effects in humans. A variety of genotoxic effects in numerous organisms from prokaryotes to mammals are known (see Table I for a summary). On account of poor data, carcinogenicity (in rats) is doubtful (Bryan, 1978). For all these reasons, a safer and also cheaper substitute for nfx is needed, because Chagas' disease has enormous social and economical implications in America.

Ten new derivatives structurally related to nfx have been synthesized from 5-nitrofurfural and the corresponding *N*-amino heterocyclic compounds and have been tested for trypanocidal activity against *T.cruzi* as potential alternative antichagasic drugs (Mester *et al.*, 1987). Compounds in which the heterocyclic moieties are of the 1,2,4-triazol-4-yl and pyridin-1-yl group clearly show higher activity against *T.cruzi* than those of the 4-thiazine-1,1-dioxide (nfx) and adamantane group. The purpose of the present work is to assess the possible genotoxicity of eight of the new derivatives and nfx as a reference in an eukaryotic test system.

The mutagenicity and possible carcinogenicity of nitrofurans may be due to the presence of two potentially reactive functional groups: the 5-nitro group and the heterocyclic substituent at the 2-position of the furan ring. The basic requirement for mutagenicity of nfx in bacterial systems is the reduction of the 5-nitro compound by at least three nitrofuran reductases (McCalla, 1983) followed by cleavage to the open chain nitrile. This is supported by the fact that nitroreductase-deficient bacteria are resistant to the lethal and mutagenic action of nfx (Nagel,

Table I. Summary of results obtained in genotoxicity tests with nifurtimox

Test system	Results	References
S.typhimurium		
His$^+$ reversion (TA100)	+ (S9=)	Ferreira and Ferreira, 1986.
His$^+$ reversion (TA100)	+ (S9−)	Nagel and Nepomnaschy, 1983.
His$^+$ reversion (TA100)	+	Ebringer and Bencova, 1980; Nagel, 1987.
His$^+$ reversion (UTH8414)	±	Nagel, 1987.
Ara test (BA13)	+ (S9−)	Alejandre-Duran *et al.*, 1988.
His$^+$ reversion (TA100, Nfz resistant)	− (S9=)	Nagel, 1987.
His$^+$ reversion (TA98, TA1535, TA1537, TA1538)	− (S9=)	Nagel and Nepomnaschy, 1983.
His$^+$ reversion (TA1535, TA1538)	−	Ebringer and Bencova, 1980.
Escherichia coli		
Arg$^+$ reversion	+	Ohnishi *et al.*, 1980.
Allium cepa		
Chromosome breaks and decreased mitotic index	+	Spano and Takahashi, 1981.
Drosophila melanogaster		
Sex-linked recessive lethals	+	Spano and Takahashi, 1981.
Mouse		
Micronuclei	+	Navarro *et al.*, 1984; Gorla and Castro, 1985.
Sperm-head abnormalities	+	Navarro and Nagel, 1984.
Rat		
Chromosomal aberrations	+	Spano and Takahashi, 1981.
Carcinogenesis	?	Bryan, 1978.
Human		
Chromosomal aberrations (lymphocytes)	+	Navarro *et al.*, 1984.

−: negative results (non-genotoxic).
+: positive results (genotoxic).
S9−: addition of S9 mixture decreased the mutagenicity.
S9=: addition of S9 mixture did not affect the results.
?: doubtful results, not very extensively studied.

1987). Another point to be taken into account is that excision repair enzymes are involved in the processing of lesions induced by nfx (Nagel, 1987). Nitroreduction and ring opening also occur in mammalian systems involving a variety of enzyme activities like xanthine oxidase, NADP-cytochrome C reductase and aldehyde oxidase (McCalla, 1983). Docampo and Stoppani (1979) have suggested that the specific activity of nfx against *T.cruzi* may be mediated by active oxygen species. The formation of nitrofuran free radicals may result in oxygen-derived metabolites (Docampo *et al.*, 1981; Moreno *et al.*, 1984; Moreno and Docampo, 1985) which have been proposed to be responsible, in part, for the cytotoxic effects of nfx.

The variable results obtained in the different bacterial mutagenicity test systems with 5-nitrofurans (Alejandre-Duran *et al.*, 1988; Ferreira and Ferreira, 1986; Nagel and Nepomnaschy, 1983; Ohnishi *et al.*, 1980) motivated us to choose an *in vivo* eukaryotic system, taking into account that the new drugs are intended for use in humans. Furthermore, genotoxic activity observed in bacteria always needs confirmation in eukaryotic cells (Ashby, 1986). *Drosophila melanogaster* is a good candidate for this purpose. The standard germline assay for sex-linked recessive lethals (SLRL) is well validated. However, it has particular disadvantages with respect to time requirements, high numbers of tests needed, assay sensitivity, etc. The role of this test system in the evaluation of potential mammalian genotoxins has been discussed recently by Vogel (1987). We decided to use the somatic mutation and recombination test (SMART) (Graf *et al.*, 1984; Würgler and Vogel, 1986) in wing cells because it allows to monitor different genetic endpoints: somatic mutation and mitotic recombination. This wing spot test is already validated with ~200 chemicals and complex mixtures so far. The possibility to detect recombinogenic activity is important because of a possible connection with the carcinogenicity of different compounds (Cairns, 1981). The inexpensive and rapid performance of the wing spot test made this somatic test system the more adequate for our purpose.

Materials and methods

Chemical compounds

All the chemical compounds tested for mutagenicity have in common the 5-nitrofurfural ring and differ in the *N*-aminoheterocyclic side chain. The compounds named 1B [1-((5-nitrofurfurylidene)amino)pyrazole], 1D [1-((5-nitro-furfurylidene)amino)-1,2,4-triazole], 1E [1-((5-nitrofurfurylidene)amino)-benzimi-dazole], 1G [1-((5-nitrofurfurylidene)amino)indazole], 1H [3,5-bis-(methylthio)-4-((5-nitrofurfurylidene)amino)-1,2-4-triazole], 1I [1-methyl-3-methylthio-4-((5-nitrofurfurylidene)amino)-1,2,4-triazole], 1K [1-((5-nitrofurfurylidene)amino) 4,6-diphenylpyridine-2-one] have previously been described by Mester *et al.* (1987). The compound ada [1-((5-nitrofurfurylidene)amino)adamantane] as well as the former seven were all synthesized and made available to us by Drs D.Sanz and R.Claramunt (Dep. Quimica Organica, Fac. Ciencias, UNED, Madrid, Spain) and Dr. B.Mester (Univ. de la Republica, Montevideo, Uruguay). Nifurtimox [1-((5-nitrofurfurylidene)amino)-3-methyl-thiomorpholine-1,1-dioxide; CAS No. 23256-30-6] was also kindly provided by Dr R.Claramunt. The abbreviations, molecular weights and structural formulae are found in Figure 1. Further analytical data of these compounds are found in Mester *et al.* (1987).

All the compounds were dissolved in 5% Tween 80 (Serva, Heidelberg, FRG) together with 5% ethanol (Merck, Darmstadt, FRG).

Larval feeding

Eggs from a cross of optimally fertile flies (see below) were collected for 8 h in culture bottles containing standard cornmeal-sugar-agar medium with extra live yeast. On day 3, the larvae were collected from the bottles using a 20% sodium chloride solution. The 3-day-old larvae were then put into plastic vials containing a special instant medium: the solutions of the various compounds were used to prepare Drosophila Instant Medium (Formula 4-24, Carolina Biological Supply Co., Burlington, NC, USA). 2 ml of solution were always added to an equal volume of powdered Instant Medium in a vial. Negative water and solvent

Abbreviation	Molecular formula (mw)	Structural formula
1B	$C_8H_6N_4O_3$ (206)	
1D	$C_7H_5N_5O_3$ (207)	
1E	$C_{12}H_8N_4O_3$ (256)	
1G	$C_{12}H_8N_4O_3$ (256)	
1H	$C_9H_9N_5O_3S_2$ (299)	
1I	$C_9H_9N_5O_3S_2$ (299)	
1K	$C_{22}H_{15}N_3O_4$ (385)	
nfx	$C_{10}H_{13}N_3O_5S$ (287)	
ada	$C_{15}H_{16}N_2O_3$ (272)	

Fig. 1. Abbreviations, molecular weights and structural formulae of the compounds tested.

controls were included. The larvae were fed on this medium for the rest of their development (~48 h until pupation).

Somatic mutation and recombination test

The following cross of flies carrying markers on the left arm of chromosome 3 was used: *mwh* females mated to *flr³/In(3LR)TM3, Ser* males. Detailed information on the genetic markers is given by Lindsey and Grell (1968). The mutation *flr³* is described by Garcia-Bellido and Dapena (1974). The surviving flies were collected from the treatment vials on days 10−12 after egg laying. Flies of the trans-heterozygous (*mwh + / + flr³*) genotype were stored in 70% ethanol. For the analysis, wings were mounted in Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and water 50 ml) and were scored under a compound microscope at 400× magnification for the occurrence of mosaic spots. Different types of spots were recorded separately, namely single spots showing either the multiple wing hairs (*mwh*) or the flare (*flr*) phenotype, and twin spots showing adjacent *mwh* and *flr* areas. Single spots are produced either by mitotic recombination between the two markers or by somatic gene mutation, deletion or other change at one or the other marker locus; twin spots are produced exclusively by mitotic recombination between the proximal marker *flr* and the centromere. In each case, the size of a spot was determined by counting the number of wing cells exhibiting the mutant phenotype. A detailed description of the wing spot test is given by Graf *et al.* (1984).

Table II. Summary of results obtained in the Drosophila wing spot test with nine antiparasitic drugs

Compound Concentration (mM)	Number of wings	Spots per wing (number of spots) diagnosis[a]				Spots with mwh clone	Mean number of cell division cycles	Frequency of clone formation $\times 10^{-5}$	
		Small single spots (1-2 cells) [m = 2]	Large single spots (>2 cells) [m = 5]	Twin spots [m = 5]	Total spots [m = 2]			Observed	Control corrected
Control (5% Tween−5% ethanol)									
0.0	295	0.20 (59)	0.06 (18)	0.02 (6)	0.28 (83)	82	2.26	1.1	
Compound ada									
0.5	85	0.41 (35)+	0.06 (5)−	0.02 (2)i	0.49 (42)+	42	1.74	2.0	0.9
1.0	81	0.25 (20)−	0.12 (10)−	0.05 (4)i	0.42 (34)+	33	2.61	1.7	0.5
5.0	34	1.44 (49)+	0.32 (11)+	0.26 (9)+	2.03 (69)+	68	2.10	8.2	7.1
10.0	20	2.00 (40)+	0.95 (19)+	0.60 (12)+	3.55 (71)+	70	2.47	14.4	13.2
20.0	20	1.95 (39)+	0.80 (16)+	1.75 (35)+	4.50 (90)+	88	2.80	18.1	16.9
Nifurtimox									
0.5	130	0.26 (34)−	0.10 (13)−	0.03 (4)i	0.39 (51)w	51	2.49	1.6	0.5
1.0	126	0.33 (41)+	0.10 (12)−	0.04 (5)i	0.46 (58)+	57	2.11	1.9	0.7
5.0	86	0.31 (27)+	0.19 (16)+	0.07 (6)+	0.57 (49)+	48	2.42	2.3	1.1
10.0	115	0.66 (76)+	0.24 (28)+	0.09 (10)+	0.99 (114)+	112	2.12	4.0	2.9
20.0	48	0.71 (34)+	0.31 (15)+	0.15 (7)+	1.17 (56)+	56	2.27	4.8	3.6
Compound 1B									
10.0	228	0.34 (77)+	0.12 (27)w	0.06 (14)+	0.52 (118)+	117	2.15	2.1	1.0
20.0	175	0.61 (107)+	0.14 (25)w	0.06 (11)+	0.82 (143)+	142	2.06	3.3	2.2
Compound 1D									
10.0	296	0.26 (77)−	0.05 (16)−	0.01 (4)−	0.33 (97)−	97	1.89	1.3	0.2
20.0	211	0.25 (52)−	0.09 (18)−	0.04 (8)i	0.37 (78)w	76	2.14	1.5	0.3
Compound 1E									
10.0	148	0.30 (44)+	0.06 (9)−	0.03 (4)−	0.39 (57)w	55	1.98	1.5	0.4
20.0	166	0.19 (31)−	0.03 (5)−	0.07 (11)+	0.28 (47)−	47	2.47	1.2	0.0
Compound 1G									
10.0	176	0.24 (42)−	0.10 (18)−	0.02 (3)−	0.36 (63)−	59	2.39	1.4	0.2
20.0	92	0.21 (19)−	0.05 (5)−	0.02 (2)−	0.28 (26)−	26	2.08	1.2	0.0
Compound 1H									
10.0	184	0.26 (48)−	0.03 (6)−	0.04 (7)i	0.33 (61)−	61	1.93	1.4	0.2
20.0	160	0.23 (37)−	0.08 (12)−	0.00 (0)−	0.31 (49)−	49	2.00	1.3	0.1
Compound 1I									
10.0	146	0.25 (36)−	0.03 (4)−	0.02 (3)−	0.29 (43)−	42	1.62	1.2	0.0
20.0	136	0.24 (33)−	0.13 (17)w	0.04 (5)i	0.40 (55)w	53	2.42	1.6	0.5
Compound 1K									
10.0	184	0.21 (38)−	0.04 (7)−	0.02 (4)−	0.27 (49)−	49	2.04	1.1	0.0
20.0	184	0.21 (39)−	0.08 (15)−	0.02 (4)−	0.32 (58)−	57	2.18	1.3	0.1

[a]Statistical diagnosis according to Frei and Würgler (1988):
m = multiplication factor; + = positive; − = negative; w = weak; i = inconclusive.

Data evaluation and statistical analysis

The evaluation of the wing spot data was performed with the computer programme SMART (Würgler, unpublished). For the statistical analysis, the spots as described above were grouped into three different categories: (i) small single spots (one or two cells in size), (ii) large single spots (three or more cells) and (iii) twin spots. These three categories of spots were evaluated separately. More details on the statistical analysis are given in Frei and Würgler (1988). For the calculations, the Kastenbaum−Bowman test was used with $P = 0.05$. Based on the number of wings analysed, the number of *mwh* clones and the number of cells scored in each wing (24'400), it is possible to calculate the clone formation frequency per cell cycle and 10^5 cells (see also Frei and Würgler, 1988).

Results and discussion

Each of the nine compounds tested was assayed in at least two independent experiments using two or more different concentrations. All compounds were tested simultaneously to obtain quantitative data which allow for a comparative analysis. Before evaluating the gentotoxicity of the various compounds, it is worthwhile to consider also the toxicity of these compounds. All the experiments were performed with concurrent water and solvent controls. The combination of 5% Tween 80 and 5%

ethanol in the solvent did not lead to measurable toxicity during the 48 h chronic feeding. For solubility reasons the highest concentration of the compounds tested was 20 mM (concentration of the solution used to prepare the Instant Medium). Nfx and ada were the most toxic of all compounds. With these two compounds the 20 mM concentration corresponded to an approximate LD_{80}. In addition, there were noticeable effects on the development of the larvae and pupae, i.e. pupation of underdeveloped larvae and late hatching of the flies. The lower concentrations of these compounds were less toxic. The other compounds were tested at 10 and 20 mM concentrations only. These treatments were also toxic, but much less than with nfx and ada, and there was no delay in development. From all these observations, it can be concluded that the different compounds were taken up by the larvae in a comparable and reproducible way during the 48 h chronic feeding. Therefore, lack of muta-genicity observed with the majority of the compounds is not due to lack of uptake of these.

For the evaluation of the genotoxic effects, the statistical treatment of the data as developed by Frei and Würgler (1988) is used here. The pooled results and the statistical diagnoses are shown in Table II. For the determination of the spontaneous mutation frequency, wings of the solvent control only were analysed for spots. Based on the frequency of total spots per wing, the spontaneous mutation rate of 0.28 is comparable to other control data obtained with these solvents (Würgler et al., 1985). From the data given in Table II, it is evident that ada and nfx were clearly mutagenic at concentrations between 0.5 mM and 20 mM. The corresponding dose response curves are plotted in Figure 2. Based on the frequency of clone formation, ada was more genotoxic than nfx by a factor of ~4.5. For this reason, more wings were analysed for nfx than for ada. Compound 1B was also clearly mutagenic, however it was much less effective than the former two. When considering the three categories of spots (i.e. small singles, large singles and twins) separately for the compounds ada, nfx and 1B, it is evident that all of them gave a positive result at least at the higher two concentrations.

For the remaining six compounds the statistical evaluation leads to the following conclusions: Compounds 1G, 1H and 1K are negative for all three categories of spots, i.e. they are non-genotoxic. Compounds 1D, 1E and 1I gave a weak effect at one of the two concentrations tested. This means that the frequencies of total spots per wing were significantly increased over the control but remained significantly below a doubling of the spontaneous mutation rate which we require to judge a result positive. When analysing the three categories of spots separately in these three series, no consistent pattern of effects is evident: In each case, only one category gave a weak or positive result. From this, it is concluded that these outcomes were due to chance variation. Our experience gained with this type of statistical evaluation has shown so far that for a positive diagnosis, the results of at least two of the three categories should be positive. It seems likely, therefore, that the compounds 1D, 1E and 1I are also non-genotoxic.

A more detailed analysis of the data obtained with the three positive compounds allows to draw further conclusions. Particularly the significant increases in twin spots demonstrate that the three compounds are definitely recombinogenic because this type of spot is produced by mitotic recombination exclusively. In contrast, the large single spots are due to various initial events: point mutation and deletion as well as mitotic recombination between the two markers can lead to this type of spot. The ratios of the frequencies of twins to large singles in the control and
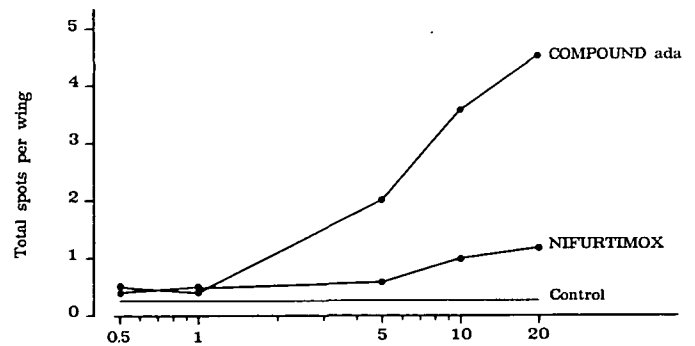

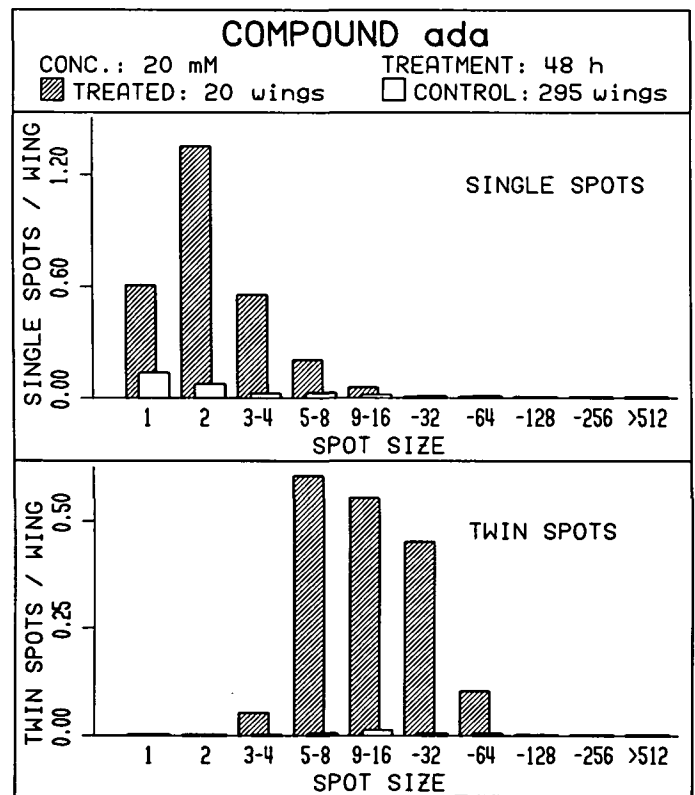Fig. 2. Dose response curves obtained in the wing spot test of D.melanogaster with nifurtimox and compound ada.

Fig. 3. Spot size distributions obtained in the wing spot test of D.melanogaster with compound ada.

the 20 mM series are as follows: control 0.33 (0.02/0.06), 1B 0.42 (0.06/0.14), nfx 0.48 (0.15/0.31) and ada 2.19 (1.75/0.80). These values indicate that the compound ada is much more recombinogenic than the other two.

For three selected data sets the spot size distributions are plotted in Figures 3, 4 and 5. A comparison of the two positive results with ada (20 mM) and 1B (20 mM) shows that with the former compound a shortage of the smallest class of spots (1 cell) is observed. The same phenomenon is also observed with nfx (data not shown). This effect is probably due to the high toxicity of these treatments which has an effect on the development of the larvae. It is possible that the larvae stop feeding prematurely and consequently the final rounds of cell division take place when the concentration of the active mutagenic species has dropped already. Similar effects have already been observed with other
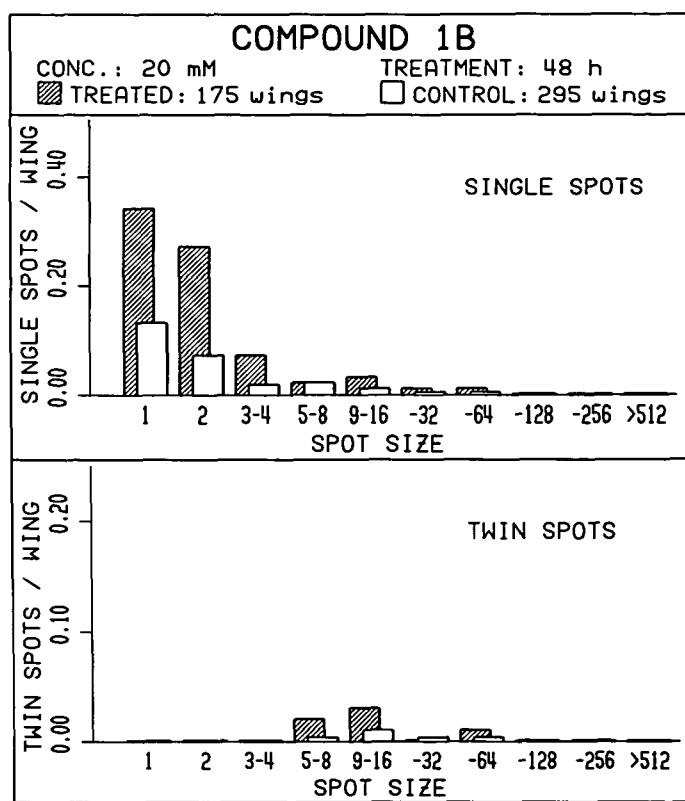
Fig. 4. Spot size distributions obtained in the wing spot test of *D.melanogaster* with compound 1B.
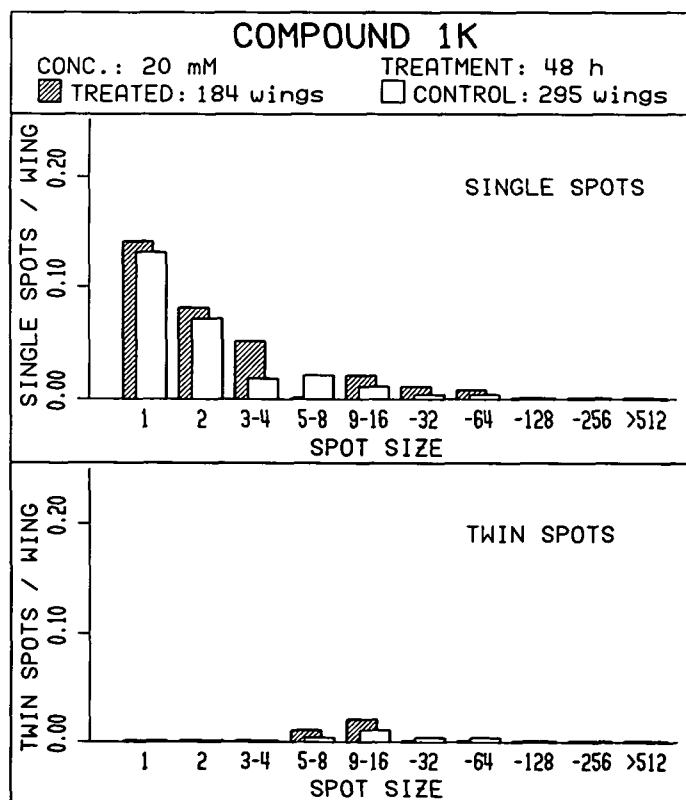

Fig. 5. Spot size distributions obtained in the wing spot test of *D.melanogaster* with compound 1K.

mutagens (Frei and Würgler, 1986). In contrast, the 20 mM treatment with compound 1B which is much less toxic, gives the expected spot size distribution (Figure 4). As an example of the negative compounds, the spot size distribution obtained with compound 1K (20 mM) shows that it is coincident with the distribution obtained with the solvent control (Figure 5).

All three mutagenic compounds produced large single spots of sizes up to $64 = 2^6$ cells. Based on the fact that one round of cell division in the wing imaginal disc lasts ~10–12 h, the largest spots correspond to mutagenic events taking place at the beginning of the third larval instar. This corresponds to the beginning of the treatment. Therefore, it can be concluded that the larvae take up these compounds and metabolize them efficiently and quickly. The positive results obtained demonstrate that the metabolic activity required for mutagenicity of these compounds is present in sufficient amounts in the Drosophila larvae. It can be assumed that the situation is similar for the compounds which gave negative results. This means that with these compounds not enough of possible mutagenic metabolites or none at all were produced.

When comparing the genotoxic effects observed in our experiments with the results obtained in other test systems, nfx confirms previous positive results (see Table I). In particular, the mutagenicity of nfx observed in germ cells of Drosophila (sex-linked recessive lethals, Spano and Takahashi, 1981) is also manifested in somatic cells. Ada and 1B are newly synthesized chemical compounds for which no mutagenicity data are available with the exception of positive results obtained in the *ara* test in *Salmonella typhimurium* (Alejandre-Duran *et al.*, 1988). However, in that test system, compound 1B was more mutagenic than ada which contrasts with the situation encountered in the wing spot test in Drosophila. In addition, all the remaining compounds were more or less active in the *ara* test, whereas they are non-genotoxic in the somatic cells of the wing.

The mutagenicity of the nitrofurans observed in bacterial test systems is most probably due to nitroreductase activity (Alejandre-Duran *et al.*, 1988; Ferreira *et al.*, 1988; Ni *et al.*, 1987). These enzymes metabolize the nitrofuran moiety of the molecule which is identical for all the compounds. The addition of S9 mixture has no major effect on the mutagenicity. Most probably, the situation encountered in such a procaryotic *in vitro* test system does not reflect the metabolic pathways present in an eukaryotic *in vivo* assay. It may be assumed that the nitroreductase activity is of lesser importance in Drosophila. This is suggested by the fact that six of the compounds are negative in the Drosophila wing spot test, although they have the same basic nitrofuran structure as the three compounds which are positive. Therefore, other metabolic pathways could be present which are able to metabolize the aminoheterocyclic side chain of these compounds.

With the exception of ada, all the compounds have been tested for antiparasitic activity in *T.cruzi* (Mester *et al.*, 1987). Nfx is among the least effective compounds, whereas compounds 1H, 1I, and 1K are the most effective antiparasitics. These compounds seem to be good candidates for the substitution of nfx if we consider that to the best of our knowledge they are non-genotoxic in Drosophila. Although its antiparasitic activity is not yet known, the newly synthesized compound ada is not very promising because of its high mutagenicity and recombinogenicity herewith documented. However, the compounds which are non-genotoxic in Drosophila all need further testing in other eukaryotic genotoxicity test systems for a final assessment.

In conclusion, the screening of these nine 5-nitrofuran compounds in the Drosophila wing spot assay has shown that this *in vivo* somatic mutation and recombination test is suited for this class of compounds and that it allows for a differentiated evaluation of potential antiparasitic drugs with respect to genotoxicity.

Acknowledgement

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