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ASPERGILLUS NIDULANS AS A TEST ORGANISM FOR THE DETECTION OF CHEMICALLY-INDUCED MITOTIC CROSSING-OVER AND CHROMOSOME MALSEGREGATION

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Summary. - *The genetic systems developed in the mould* Aspergillus nidulans *to study the chemical induction of mitotic genetic segregation were used to investigate the mechanism of aneuploidy induction by the main benzene hydroxy metabolites. Detailed genetic analysis of mitotic segregants produced by individual colonies revealed the simultaneous occurrence of both whole chromosome segregants and mitotic cross-overs, i.e. a pattern not compatible with the induction of numerical abnormalities as the primary event. Confirmatory evidence was obtained in a haploid strain, thus demonstrating the possible secondary origin of aneuploidy following the induction of structural chromosome damages. Furthermore, mutagenic, lethal and growth-arresting properties of a series of chlorinated hydrocarbons were quantitatively estimated and compared to a series of physico-chemical descriptors of the molecules to assess the possible role of unspecific physical interactions in their mechanism of action. The analysis of the interrelationships among these variables highlighted a possible correlation among physico-chemical descriptors and toxic properties and a dissociation of the aneugenic activity with the other chemical and biological descriptors.*

Riassunto (L'impiego di *Aspergillus nidulans* per il rilevamento di aneuploidia e ricombinazione mitotica indotta chimicamente). - *I sistemi genetici messi a punto nel fungo* Aspergillus nidulans *per lo studio della segregazione mitotica sono stati utilizzati per analizzare il meccanismo d'azione dei principali metaboliti idrossilati del benzene. L'analisi del pattern di segregazione prodotto da singole colonie dopo trattamento chimico ha rivelato in un numero consistente di casi la segregazione contemporanea di non-disgiunzionali ed altri aneuploidi insieme a ricombinanti mitotici, in modo non compatibile con l'ipotesi della induzione di anomalie cromosomiche numeriche come effetto primario. Test su un ceppo aploide hanno dimostrato l'assenza di veri iperploidi dopo trattamento con idrossibenzeni dimostrando che gli aneuploidi individuati nel diploide hanno origine secondaria in seguito alla induzione ed alla evoluzione di danni cromosomici strutturali. E' stata inoltre determinata l'attività aneuploidizzante, tossica e citostatica di una serie di idrocarburi alifatici clorurati per indagare il possibile coinvolgimento di interazioni aspecifiche, dipendenti dalle proprietà lipofile, nel loro meccanismo d'azione. L'analisi delle correlazioni tra caratteristiche biologiche e descrittori chimico-fisici ha permesso di individuare una relazione tra lipofilicità, tossicità e capacità di arrestare la mitosi, evidenziando inoltre una dissociazione tra l'attività aneuploidizzante, gli altri effetti tossici e le caratteristiche chimico-fisiche considerate.*

Introduction

The mould *Aspergillus nidulans (A. nidulans)* is currently used as a test organism in various fields of biological research, such as molecular biology, where genetic transformation and recombinant vectors are extensively used in gene cloning, cell biology, formal genetics and environmental mutagenesis [1]. The life cycle of *A. nidulans* can be divided in a meiotic cycle, in which sex organs and ascospores are formed and a vegetative cycle in which only mitotic divisions occur. According to the requirement of the researcher therefore both meiotic or mitotic uninucleated cells can be obtained for various purposes. Another major advantage in the use of *A. nidulans* in genetic toxicology is the availability of both haploid and diploid stable strains. Stable diploids are formed in the "parasexual cycle" [2], a mechanism which provides some genetic variability even in the absence of meiosis through the occurrence of mitotic segregation phenomena such as mitotic crossing-over or chromosome misdistribution [3]. These genetic events, either spontaneous or chemically-induced, can be studied in detail in *A. nidulans* by genetic procedures which are widely used in environmental mutagenesis to identify chemicals able to affect

mitotic genetic segregation [4]. These procedures are selective or unselective depending on the genetic marker used and offer different advantages. Unselective methods are particularly suitable for studies on chromosome malsegregation (aneuploidy) induction, as unbalanced aneuploid types are usually less viable than euploid types and may be not recovered in the presence of partially inhibiting concentrations of selective agents. On the other hand, selective methods are very suitable to detect chemically-induced mitotic recombination which produces euploid (diploid) segregants with normal viability.

The data base of *A. nidulans* mitotic recombination and aneuploidy assays was recently reviewed [5, 6]. These analysis demonstrated that mitotic crossing-over induction in *A. nidulans* is a sensitive indicator of DNA damaging activity, suitable to detect electrophilic reactants. Furthermore it was shown that a significant fraction (about one third) of the chemicals evaluated as positive in *A. nidulans* aneuploidy assays, amongst which several "false negative" carcinogens [7], do not induce any other genetic event, thus suggesting the involvement of targets other than DNA in their mechanism of action.

In our laboratory we have recently used the methodologies previously set up in *A. nidulans* to investigate two aspects of relevance in the mechanism of aneuploidy induction, i.e. the nature of the primary genetic event induced and the specificity of the mechanism(s) involved. The results of this study are summarized and briefly discussed in this paper.

Materials and methods

Strains and genetic systems

The *A. nidulans* diploid strains 19 (*fpaB37, galD5, riboA1, anA1, yA2, adE20, biA1; acrA1; actA1; pyroA4; facA303; sB3; choA1; fwA2, chaA;*) and P1 (*suA1adE20, riboA1, fpaA1, anA1, proA1, pabaA1, yA2, adE20, biA1; methA1, pyroA4; nicA2; lysB5; nicB8;*) and the haploid strains 35 (*anA1, pabaA1, yA2; methG1; s12; nicA2; nicB8;*) were used throughout the work.

Mitotic segregants in diploid P1 were detected as homozygous (yA2/yA2) or hemizygous (yA2/0) yellow sectors or patches in heterozygous (yA2/yA$^+$) pale-green colonies growing on complete medium. Yellow segregants were isolated, analyzed for their nutritional requirements and classified as mitotic cross-overs, non-disjunctional diploids and haploids as previously described [8].

Mitotic segregants in diploid 19 were analyzed by "needle plating" [9]. Briefly, abnormal colonies showing segregation of *yA2* marker were replated to provide at least 12 secondary euploid yellow segregants from each replated abnormal colony. Induced aneuploidy was distinguished from chromosome damage on the basis of the pattern of segregation of abnormal colonies, aneuploid types producing euploid sectors only by segregation of whole chromosomes and other unbalanced types producing diploid sectors by crossing-over as well as by whole chromosome segregation.

Abnormal, presumptive aneuploid colonies in haploid strain 35 were visually identified among colonies growing on complete medium and analyzed by "needle plating" to distinguish aneuploid, unstable types producing normal vigorous sectors from stable abnormals, presumably mutant types.

Treatments with test chemicals

Conidia of haploid and diploid strains were preincubated 3 h 30 min in semi-liquid (agar 0.15%) complete medium at 37 °C with gentle shaking. Samples of swollen, pregerminating conidia were treated with the chemical under test until the emergence of germ tubes (usually 3 h). Treatments were stopped by serial dilutions with sterile distilled water and conidia plated at low densities (to provide 10-15 colonies per plate) on agarized complete medium.

Results and discussion

The mechanism of induction of aneuploidy by the major hydroxylbenzene metabolites was studied. Hydroquinone, catecol and phenol all proved to significantly increase the frequency of whole chromosome segregants in *A. nidulans* strain 19 (Table I). Hydroquinones are known to interfere with tubulin polymerization *in vitro* and to produce by autooxidation reactive semiquinones capable of inducing structural chromosome damages as well. Therefore, a genetic analysis was carried out to disclose whether aneuploidy was primarily induced or if it resulted as a secondary event following the induction of structural chromosome aberrations which can be eliminated through subsequent genetic recombination or chromosome malsegregation. For this purpose, the pattern of segregation produced by individual abnormal colonies induced by hydroquinones was studied by "needle plating". Data in Table 2 demonstrate that in a significant proportion of cases (17 out of 80) *both* non-disjunctionals (e.g. whole chromosome segregants) and cross-overs (and/or terminal deletions) were produced. Conversely, in the case of the fungicide benomyl - an agent known to interfere primarily with chromosome segregation - only whole cromosome segregation was induced. This analysis suggests therefore that the primary genetic event induced by hydroquinones was not aneuploidy but another kind of genetic damage eliminated by the cell through secondary recombination and chromosome segregation. Confirmatory evidence supporting this view was provided by experiments with haploid strain 35 (Table 3). Experiments with haploids can demonstrate unambiguously the primary origin of aneuploidy as those large structural chromosome damages (rearrangements, deletions) which in diploids can bring to aneuploidy as a secondary event are expected to be lethal in haploids. Accordingly, hydroquinones only

Table 1. - *Induction of mitotic segregation by hydroxybenzenes in* A. nidulans *diploid strain 19*

Compound	Concentration (mM)	Scored colonies	Survival (%)	yA segregants			
				non-disjunctionals *(a)*		cross-overs *(b)*	
				no.	(%)	no.	(%)
hydroquinone	0	2311	100	2	0.1	12	0.5
	0.5	917	88	2	0.2	4	0.4
	1	1379	85	7	0.5 *(*)*	10	0.7
	2	2106	60	13	0.6 *(**)*	13	0.6
	3	1409	29	11	0.8 *(**)*	11	0.8
catechol	0	1036	100	3	0.3	6	0.6
	5	474	96	2	0.4	0	
	10	420	85	6	1.4 *(**)*	4	1.0
	15	834	72	13	1.6 *(**)*	7	0.8
	20	443	69	5	1.1 *(**)*	1	0.2
phenol	0	776	100	0		3	0.4
	5	265	100	0		3	0.8
	10	738	100	1	0.1	3	0.4
	15	826	95	5	0.6 *(*)*	2	0.2
spontaneous control *(c)*		4123	100	5	0.1	21	0.5
positive control *(d)*		147	28	27	18.4*(**)*	1	0.7

(a) $fpa^{r}gal^{-}ribo^{-}ade^{+}$; *(b)* $fpa^{s}gal^{+}ribo^{+}ade^{-}$; *(c)* cumulative values; *(d)* benomyl 1.7 μM; *(*)* $p < 0.05$;*(**)* $p < 0.01$ (χ^2 test)

Table 2. - *Characterization of yA abnormals of* A. nidulans *diploid strain 19 by "needle plating"*

no. of yA abnormal colonies segregating:	Treatment: None	Hydroquinone (1-3 mM)	Catechol (10-15 mM)	Phenol (15 mM)	Benomyl (1.7 μM)
a) only non-disjunctional *(a)* sectors	4	18	13	11	18
b) only cross-over *(b)* sectors	1	5	4	3	0
c) both types	0	6	5	6	0
d) others *(c)*	0	5	2	2	0

(a, b) see legend of Table 1; *(c)* complex phenotypes such as $fpa^{r}gal^{-}su^{-}ribo^{-}an^{-}$, $fpa^{r}gal^{-}su^{-}ribo^{+}$, $fpa^{s}gal^{+}su^{+}ribo^{+}bio^{-}$ and $fpa^{s}gal^{+}su^{+}ribo^{+}$

Table 3. - *Induction of abnormal colonies in* A. nidulans *haploid strain 35 (treatment with hydroquinone)*

Concentration (mM)	Survival (%)	Scored colonies	Abnormal colonies			
			unstable		stable	
			no.	(%)	no.	(%)
0	100	716	3	0.4	3	0.4
0.5	100	475	6	1.3	4	0.8
1	87	629	3	0.5	14	2.2 *(*)*
2	80	799	6	0.8	19	2.4 *(*)*
Positive control *(a)*	31	113	11	9.7 *(*)*	0	

(a) benomyl 1.7 μM; *(*)* $p < 0.01$ (Fisher's test)

proved to increase stable abnormals types (presumably mutants), whereas no increase in unstable abnormals, putative aneuploids producing vigorous wild type segregants was observed (Table 3).

Another question which was addressed in this study concerns the specificity of the aneuploidy-inducing activity of chemical agents. Most lipophilic chemicals in fact were shown to induce chromosome malsegregation as the primary event in *A. nidulans* and in other *in vitro* genetic systems. Therefore the question can be raised as to whether the disturbance of mitotic chromosome segregation is due to the chemical disturbance of specific cellular targets/mechanisms, or whether the effects observed are consequence of non-specific interactions based on the partitioning of lipophilic compounds into hydrophobic cellular compartments (e.g. biological membranes). Such unspecific mechanism was shown to work in the case of the chemical induction of c-mitosis and anesthesia which are directly related to descriptors of lipophilicity such as the partition coefficiency octanol/water [10]. To clarify

this point, which is of major relevance for the evaluation and extrapolation of *in vitro* experimental results, the interrelationships among a few physico-chemical descriptors and indicators of aneuploidy induction and cell toxicity were investigated in a series of structurally-related halogenated hydrocarbons.

Fifteen chlorinated methanes, ethanes and ethylenes were studied in the *A. nidulans* mitotic segregation system employing the diploid strain P1. The ability to induce chromosome malsegregation (quantified by the lowest effective concentration (LEC) and maximum fold increase over spontaneous rates (INCR)), and the toxicity (expressed by the dose associated with 37% of survivors (D37), i.e. one lethal hit per cell, and by the lowest concentrations able to arrest conidial germination (ARRES)), were determined in strictly standardized conditions (Table 4).

Furthermore, the following physico-chemical descriptors were calculated or searched in the literature: molecular weight (MW), melting point (MP), boiling point (BP), refractive index (ND_{20}), partition coefficient in octanol/water (log_p), and average free energy of binding to biological receptors (ΔG). The interrelationships among these biological and chemical descriptors were investigated by means of multivariate statistical analysis. Table 5 shows the correlation matrix of the biological and chemical variables. MW, BP, ND_{20}, log_p and ΔG proved to be significantly related to each other, with r values greater than 0.90. Aneuploidizing activity (1/LEC and INCR) was poorly related to the lipophilicity of chemicals (log_p), as well as to all other chemical descriptors (r values from 0.02 to 0.26). The reverse was true for the ability to arrest mitotic division and conidial germination (ARRES), which showed r values ranging from -0.57 to -0.77. A trend for

Table 4. - *Chemico-physical and biological descriptors of chlorinated aliphatic hydrocarbons*

Chemical	Chemico-physical variables						Biological activity in *A. nidulans* aneuploidy induction		cell toxicity	
	MW	MP	BP	ND_{20}	log_p	ΔG	LEC (mM)	INCR	ARRES (mM)	D37 (mM)
dichloromethane	86.9	-100.0	40.0	1.4	1.3	-10.6	62.7	9.5	94.0	141.0
chloroform	119.4	-73.5	61.2	1.4	2.0	-9.3	20.0	4.1	25.0	27.5
carbon tetrachloride	153.8	-23.0	76.7	1.5	2.6	-8.0	4.1	10.0	10.4	7.3
1,1-dichloroethane	99.0	-97.0	57.0	1.4	1.3	-10.0	23.8	3.7	47.6	38.1
1,2-dichloroethane	99.0	-35.5	83.0	1.4	1.5	-10.5	25.4	17.1	50.8	44.4
1,1,1-trichloroethane	133.4	-35.0	75.0	1.4	1.9	-8.5	-	0	10.0	11.0
1,1,2-trichloroethane	133.4	-37.0	112.5	1.5	2.1	-9.2	8.1	8.4	10.7	11.8
1,1,1,2-tetrachloroethane	167.8	-68.1	138.0	1.5	3.0	-7.9	1.2	42.1	3.8	2.4
1,1,2,2-tetrachloroethane	167.8	-43.0	147.0	1.5	2.7	-7.9	1.9	3.0	3.8	3.3
pentachloroethane	202.3	-29.0	161.5	1.5	4.7	-6.6	-	0	1.7	1.2
hexachloroethane	236.7	-28.2	192.5	1.5	4.6	-5.3	-	0	0.8	1.7
1,1,2-trichloroethylene	131.4	-87.0	87.0	1.5	2.3	-8.7	5.5	7.0	22.0	11.0
tetrachloroethylene	165.8	-22.0	121.0	1.5	3.0	-7.4	-	0	1.2	1.5
1,2-dichloroethylene	96.9	-57.0	48.6	1.4	1.5	-10.0	16.3	9.1	32.7	26.2
1,1-dichloroethylene	96.9	-122.0	31.0	1.4	1.7	-10.0	6.3	4.1	25.0	21.3

Abbreviations. - MW: molecular weight; MP: melting point; BP: boiling point; ND_{20}: refractive index; log_p: partition coefficient in octanol/water; ΔG: free energy of binding to biological receptors; LEC: lowest efficient concentration in aneuploidy induction; INCR: maximum fold-increase over spontaneous rates; ARRES: lowest concentration arresting conidial germination; D37: concentration determining 37% of survivors.

Table 5. - *Interrelationships among physico-chemical descriptors of a series of halogenated aliphatic hydrocarbons and their biological activity in A.* nidulans. *Correlation matrix*

	MW	MP	BP	ND_{20}	log_p	ΔG	1/LEC	INCR	ARRES	D37
MW	1.00									
MP	0.62	1.00								
BP	0.93	0.62	1.00							
ND_{20}	0.89	0.65	0.90	1.00						
log_p	0.96	0.54	0.89	0.87	1.00					
ΔG	0.99	0.60	0.88	0.87	0.95	1.00				
1/LEC	0.18	-0.09	0.26	0.25	0.10	0.13	1.00			
INCR	-0.10	-0.14	0.02	-0.03	-0.11	-0.17	0.74	1.00		
ARRES	-0.75	-0.57	0.67	-0.74	-0.68	-0.77	-0.31	0.07	1.00	
D37	-0.61	-0.49	0.55	-0.62	-0.55	-0.63	-0.28	0.05	0.95	1.00

Abbreviations: see legend of Table 4

correlation with lipophilicity as well as with the other descriptors was also observed for D37 (r values from -0.49 to 0.63). If the relationships among biological descriptors are considered, it can be observed that the ability to disturb chromosome segregation is not closely related to the toxic properties of test chemicals, whereas lethal and cytostatic activities show a mutual r value of 0.83.

In conclusion the results of this analysis demonstrate a dissociation between aneugenic and toxic properties of chemical compounds and highlight the absence of a simple correlation between aneuploidy-inducing activity and lipophilicity. All this clearly argues against the involvement of non-specific interactions with hydrophobic cellular structures in the mechanism of aneuploidy induction by halogenated hydrocarbons. Rather, a role for such non-specific mechanism could be hypothesized in the case of the toxic properties exerted. Obviously, the lack of non-specific interactions in the mechanism by which organic compounds affect chromosome segregation allows one to regard with greater confidency the results obtained in *in vitro* assays which are more likely to be biased by unspecific effects.

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