Transformation of BALB/c 3T3 Cells *in vitro* by the Fungicides Captan, Captafol and Folpet

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Cytotoxic and cell-transforming activities of the three fungicides, captan, captafol and folpet, have been studied in an experimental *in vitro* model by exposing BALB/c 3T3 cells to the chemicals with or without S-9 mix-induced bioactivation. Cytotoxicity of the three compounds was reduced in the presence of the metabolizing system. Each assayed pesticide displayed cell-transforming ability in the presence of the metabolizing system. The relative efficiency was: captafol > captan > folpet. Cell transformation was considered to be due to carcinogenesis-promoting activity. These data, obtained in a medium-term (6–8 weeks) experimental model, contribute to a better understanding of the action of the three pesticides in the multistep carcinogenesis process and provide more information concerning the oncogenic risk of these xenobiotic compounds for humans.

Key words: Pesticide — Environmental risk — BALB/c 3T3 cell line — Carcinogenesis promotion — Pesticide metabolism

Captan and related compounds such as captafol and folpet have similar fungicidal properties and are widely used in horticultural and agricultural sprays to prevent fungal diseases in fruits, vegetables and flowers. Furthermore, these compounds are utilized as fungicides in household gardens and as additives for mold resistance in paints, soaps, paper, leather, etc.

As far as the oncogenic risk for humans is concerned, these chemicals are known as DNA-damaging agents, acting as mutagens in various *in vitro* experimental systems utilizing prokaryotic and eukaryotic cells[1–3] and eliciting DNA-repair synthesis.[4] However, controversial or negative data have been reported on *in vivo* mutagenic activity of these compounds on *Drosophila*,[5, 6] mice and rats.[7–11] Captan, captafol and folpet produced liver, kidney and uterus tumors in treated rodents[12–15] and they gave positive results in *in vivo* medium-term (20 weeks) bioassay utilizing rats previously treated with powerful carcinogens.[16, 17] Nevertheless, negative results were obtained for the carcinogenic activity of captan on rodents in previous *in vivo* experiments.[18–20] These discrepancies have led to different conclusions on the oncogenic risk associated with these compounds. The National (Italian) Toxicology Advisory Committee evaluated captan and folpet as possibly carcinogenic to humans.[21] However, the IARC Working Group on the Evaluation of Carcinogenic Risk of Chemicals to Humans concluded that inadequate evidence of carcinogenicity exists for animals treated with captan and no adequate data are available from epidemiological studies to define the possible onco-

genic risk of the chemical for humans.[22] But, based on the carcinogenic activity of captafol on animals, this chemical was classified as probably carcinogenic for humans.[23] It is noteworthy that these apparent differences in oncogenic potential for humans exist even though the three compounds share common metabolic pathways and have a close molecular similarity which might suggest similar mechanisms of action.
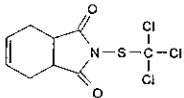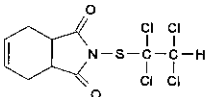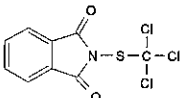
The aim of the present study was to obtain further information on the oncogenic risk of captan, captafol and folpet for humans. We used a medium-term assay (6–8 weeks) *in vitro* in order to test the cytotoxicity and cell-transforming activity of each compound on BALB/c 3T3 cells, with or without S-9-induced bioactivation.[24] This experimental model is suitable to study the carcinogenic activity of xenobiotic compounds. Previous data on the carcinogenic potential of chemicals have shown a good relationship between *in vitro* cell transformation and *in vivo* carcinogenesis with a 69 to 85% correlation.[25]

MATERIALS AND METHODS

Chemicals Chemical structures and degree of purity of the pesticides utilized in the present study are reported in Table I. Captan was purchased from the Institute of Organic Industrial Chemistry, Warsaw, Poland; captafol and folpet were from Laboratory Dr. Ehrenstorfer, Augsburg, Germany. 3-Methylcholanthrene (3-MCA) and benzo[a]pyrene (B(a)P) were obtained from Sigma, St. Louis, MO. Pesticides, 3-MCA and B(a)P were dissolved in dimethylsulfoxide (DMSO) (Riedel-De Haen,

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Table I. List of Fungicides Assayed in the Study

Common name	Chemical name	Chemical structure	CAS number	Purity (%)
Captan	N-(Trichloromethylthio)-4-cyclohexene-1,2-dicarboximide		133-06-2	99.0
Captafol	N-(1,1,2,2-Tetrachloroethylthio)-4-cyclohexene-1,2-dicarboximide		2425-06-1	99.9
Folpet	N-(Trichloromethylthio)phthalimide		133-07-3	99.8

Hannover, Germany) because of their low water solubility, and 0.5% DMSO-containing culture medium (final concentration) was utilized in chemical-treated and control cultures.

Nicotinamide adenine dinucleotide phosphate (NADP), NADP reduced form (NADPH), nicotinamide adenine dinucleotide, reduced form (NADH), d-glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase grade II were purchased from Boehringer Mannheim, Milan, Italy.

Cells, cell cultures and S-9 fraction The original stock of BALB/c 3T3 cells, clone A31, was obtained from the American Type Culture Collection, Rockville, MD. Working cultures were expanded from the original cryopreserved stock. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (Gibco, Paisley, UK) supplemented with 10% newborn calf serum (Gibco). The antibiotics streptomycin and penicillin were present at 50 μg/ml and 50 units/ml, respectively. Only subconfluent (60–70% confluent) cultures were used in the assays and target cells were not maintained beyond the third passage after thawing. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

S-9 fraction was extracted from liver of rats pretreated with phenobarbitone as reported.[26] The activating system mixture (S-9 mix) contained 50 μl/ml S-9, 2 μg/ml G-6-P dehydrogenase, 0.2 mM G-6-P, 0.28 mM NADH, 0.25 mM NADP, and 0.27 mM NADPH.

Cytotoxicity test Exponentially growing cells were seeded for each treatment at 350 cells/60-mm dish in four replicates per treatment. Twenty-four hours thereafter, pesticides, dissolved in 0.5% DMSO, were diluted in culture medium and added to cell cultures. Captan was tested at the final concentrations of 5, 1, 0.5 and 0.1 μg/ml; captafol and folpet were employed at the final concentrations of 0.5, 0.1, 0.05 and 0.01 μg/ml and 20,

10, 5 and 1 μg/ml, respectively. The final solvent concentration (0.5%) was equal for all treatments. Nagative controls (untreated cells and solvent vehicle-treated cells) were run. Following exposure, the treatment medium was removed, and cells were washed with phosphate-buffered saline (PBS) and refed with 3 ml of complete growth medium. Cells were exposed to the test compound for 72 h or for 4 h in the absence or in the presence of S-9 mix activating system, respectively. In the presence of the activating system, incubation was carried out with serum-free medium. The medium was changed twice weekly for the duration of the assay (7–10 days). At the end of this period, dishes were fixed with methanol, stained with 10% aqueous Giemsa and scored for colony formation. Only colonies larger than 50 cells were counted.

Transformation test Cell transformation assay was performed according to the recommended experimental protocol[25, 27] with some minor modifications as previously described.[26] Briefly, exponentially growing cells were seeded for each treatment at 1×10^4 cells/60 mm dish in ten replicates and cultured. Twenty-four hours thereafter, three non-toxic or weakly toxic doses of each pesticide previously utilized in the cytotoxicity test were added to the culture medium. This allowed comparison of the cytotoxic and cell-transforming activities of the chemicals at equal dosages with or without metabolic activation. The final solvent concentration (0.5%) was equal for all the treatments. Negative controls (untreated cells and 0.5% DMSO-treated cells) were run in ten replicates. Positive controls utilized 2.5 μg/ml 3-MCA or B(a)P as the transforming agent in the absence or in the presence of S-9 mix metabolizing system, respectively.

In the absence of S-9 mix, cells were exposed to the test compounds for 72 h. In the presence of S-9 mix, cells were incubated for 4 h in serum-free medium containing the metabolizing system. At the end of the incubation

period, all treatment media were removed, and cells were washed with PBS and refed with 3 ml of 10% serum-containing medium. Culture medium was changed twice weekly until confluence was attained (about two weeks). At this time, a level-II transformation test was performed in order to obtain transformation amplification. Level-II transformation plates were generated by pooling the contents of four confluent plates (level-I transformation plates defined as the standard test) and reseeding 1×10^5 cells/60 mm dish in sextuplicate dishes for each treatment. Medium was changed twice weekly in the level-I and level-II culture plates, which were fixed (methanol), stained (10% aqueous Giemsa) and scored for foci formation about six weeks after the beginning of the tests. **Scoring of foci** Only foci considered as positive,[25, 27] showing deep basophilic dense multilayering of cells, random cell orientation at any part of the focus edge, invasion into the surrounding contact-inhibited monolayer and dominance of spindle-shaped cells, were counted.

Transformation frequencies were assessed by calculating: 1) the number of positive plates (plates with foci) per scored plates; 2) the average number of foci/plate/ each treatment±standard error; 3) the transformation frequency index (TF), i.e. the total number of foci per group (each treatment) divided by the number of cells at

risk (cells surviving after each treatment) estimated from the clonal efficiency observed in the cytotoxicity assay. **Statistical evaluation** Statistical evaluation was performed by use of the Fisher Yates test and the Mann-Whitney unpaired t test, respectively, as regards the number of plates with foci/scored plates/dose and the number of foci/plate/dose.

RESULTS

Table II shows the cytotoxic effects exerted by four doses of each pesticide on BALB/c 3T3 cells in cultures performed in the absence or in the presence of S-9 mix-induced bioactivation of chemicals (±S9 mix).

In the absence of metabolic activation each compound exerted very strong cytotoxic activity at the highest tested dose, i.e. 0.5, 5 and 20 $\mu g/ml$ for captafol, captan and folpet, respectively. It is noteworthy that the presence of 10% serum in culture medium did not affect the cytotoxic potency of the compounds. This finding is inconsistent with the loss of toxicity of captan and of chemically related compounds induced by the presence of thiols, e.g. cysteine, in serum added to culture media.[28] Captafol was the most toxic agent; it showed potent toxicity at 0.5 $\mu g/ml$. Cytotoxic effects were not observed

Table II. Cytotoxic Effects of Captafol, Captan and Folpet on BALB/c 3T3 Cells in the Absence or in the Presence of an Exogenous Metabolizing System (S-9 mix)[a]

Treatment	Dose ($\mu g/ml$)	− S-9 mix			+ S-9 mix		
		Mean number of colonies/plate	Clonal efficiency[b]	Relative clonal efficiency (%)[c]	Mean number of colonies/plate	Clonal efficiency[b]	Relative clonal efficiency (%)[c]
Untreated		55.25±2.00	0.157	121	33.50±2.33	0.096	93
DMSO (0.5%)		45.50±2.36	0.130	100	36.00±0.90	0.102	100
3-MCA	2.5	36.75±2.90[f]	0.105	81			
B(a)P	2.5				27.50±2.50[d]	0.078	76
Captafol	0.5	14.50±1.00[e]	0.041	32	29.00±0.40[e]	0.083	81
	0.1	37.50±1.44[d]	0.107	82	27.00±2.68[d]	0.077	75
	0.05	42.75±2.50[f]	0.122	94	30.00±1.29[e]	0.086	83
	0.01	47.50±3.86[f]	0.135	104	30.25±3.10[f]	0.086	84
Captan	5	0.25±0.25[e]	0.001	0.55	30.50±3.50[f]	0.087	85
	1	37.25±1.84[d]	0.106	82	34.00±2.10[f]	0.097	94
	0.5	38.50±1.85[f]	0.110	85	28.50±2.72[d]	0.081	79
	0.1	43.75±0.95[f]	0.125	96	27.75±0.25[e]	0.080	77
Folpet	20	3.00±0.70[e]	0.008	7	28.75±3.10[f]	0.082	80
	10	30.75±1.55[e]	0.088	68	36.50±1.20[f]	0.104	101
	5	37.00±2.35[d]	0.106	81	27.25±1.60[d]	0.077	76
	1	40.00±2.48[f]	0.114	88	31.50±1.20[d]	0.090	88

a) Data are mean values of four culture plates/treatment±SE.
b) Clonal efficiency: number of colonies/3.5×10^2 cells seeded per plate.
c) Total number of colonies per treatment relative to the negative control (0.5% DMSO).
Significant difference from control (DMSO-treated plates) based on Student's t test: d) $P < 0.05$; e) $P < 0.01$; f) not significant.

Table III. *In vitro* BALB/c 3T3 Cell Transformation (Level-I) by Captafol, Captan and Folpet in the Absence or in the Presence of an Exogenous Metabolizing System (S-9 mix)

Treatment	Dose (μg/ml)	− S-9 mix			+ S-9 mix		
		Plate with foci/plates scored	Mean no. of transformed foci/plate	TF $(\times 10^{-4})^{a)}$	Plate with foci/plates scored	Mean no. of transformed foci/plate	TF $(\times 10^{-4})^{a)}$
Untreated		3/6	0.66±0.33	4.20	3/6	0.67±0.33	6.98
DMSO (0.5%)		3/6	0.50±0.22	3.85	2/6	0.50±0.34	4.90
3-MCA	2.5	3/6	1.17±0.54	11.14			
B(a)P	2.5				5/6	1.00±0.26	12.82
Captafol	0.1	$4/6^{c)}$	1.33±0.49	12.43	$6/6^{b)}$	$3.00\pm0.73^{d)}$	38.96
	0.05	3/4	1.50±0.64	12.30	$6/6^{b)}$	$4.00\pm0.77^{e)}$	46.51
	0.01	$4/5^{b)}$	1.20±0.37	8.89	$5/6^{c)}$	2.00±0.86	23.26
Captan	1	2/3	1.00±0.58	9.43	$6/6^{b)}$	$1.83\pm0.31^{d)}$	18.87
	0.5	$2/4^{b)}$	0.50±0.29	4.55	$4/6^{c)}$	1.00±0.36	12.34
	0.1	$3/6^{c)}$	0.50±0.22	4.00	$5/6^{c)}$	1.50±0.34	18.75
Folpet	10	0/5	0.00±0.00	0.00	$5/6^{c)}$	1.16±0.31	11.15
	5	$1/6^{b)}$	0.17±0.17	1.60	$6/6^{b)}$	$1.83\pm0.31^{d)}$	23.77
	1	$4/5^{b)}$	1.00±0.32	8.77	$3/6^{c)}$	0.83±0.48	9.22

a) Transformation frequency expressed as the number of transformed foci per number of cells at risk after chemical treatment.
b, c) Significantly different from control (DMSO-treated plates) using the Fisher Yates test of significance in 2×2 contingency tables: *b*) $P<0.05$; *c*) $P<0.01$.
d, e) Significantly different from control (DMSO-treated plates) using the distribution-free Mann-Whitney unpaired *t* test: *d*) $P<0.05$; *e*) $P<0.01$.

at 0.05 or 0.01 μg/ml of captafol, 0.1 μg/ml of captan or 1 μg/ml of folpet.

In the presence of bioactivation, the pattern of cytotoxic effects exerted by the three pesticides was quite different. In fact, the S-9 mix-induced metabolic activation of the compounds led to a marked decrease of their cytotoxic effects (see the number of colonies and the relative clonal efficiency percentages in Table II).

Results from level-I cell transformation assay are shown in Table III. Cultures were treated with three doses of each chemical, starting from the highest dose exerting weak cytotoxicity in the absence of bioactivation (0.1, 1 and 10 μg/ml for captafol, captan and folpet, respectively), either in the absence or presence of metabolic activation (±S-9 mix). In the absence of bioactivation, the mean numbers of transformed foci from cultures treated with the three pesticides were not significantly different from that of the negative control (DMSO-treated cultures). However, TF values from captafol-treated and 1 μg/ml captan-treated cultures were similar to that of the positive control (3-MCA-treated plates). The high TF value (8.77) from the cultures treated with the lowest dose of folpet (1 μg/ml) is difficult to explain: it could be coincidental.

In the presence of metabolic activation, captafol showed strong activity as a cell-transforming agent. It induced significantly higher numbers of transformed foci than the negative control (DMSO-treated cultures) at

0.1 and 0.05 μg/ml doses, and 2- to 4-fold higher TF values than the positive control (B(a)P-treated plates) at all dosages used. Captan and folpet showed lower cell-transforming activity than captafol. They induced significantly higher numbers of transformed foci compared with DMSO-treated cultures only at a single dosage (1 and 5 μg/ml, respectively) and gave TF values similar to that of the positive control.

When cells were allowed to undergo further rounds of replication (level-II amplification test) (Table IV), the pattern from the level-I cell transforming cultures (Table III) became more evident. In fact, in the absence of bioactivation, only the highest tested dose of captafol (0.1 μg/ml) induced a statistically significant increase of the number of transformed foci per plate compared with DMSO-treated cultures and a TF value >2 fold higher than that of the positive control (3-MCA-treated plates).

In the presence of bioactivation, a statistically significant, dose-related increase of the numbers of transformed foci per plate compared with that of the negative control was observed for each pesticide. Furthermore, TF values were markedly higher than that of the corresponding negative control (solvent-treated cultures).

DISCUSSION

Captan, captafol and folpet are capable of inducing *in vitro* transformation of BALB/c 3T3 cells. Transforming

Table IV. *In vitro* BALB/c 3T3 Cell Transformation (Level-II Amplification) by Captafol, Captan and Folpet in the Absence or in the Presence of an Exogenous Metabolizing System (S-9 mix)

Treatment	Dose (μg/ml)	− S-9 mix			+ S-9 mix		
		Plate with foci/plates scored	Mean no. of transformed foci/plate	TF ($\times 10^{-4}$)[a]	Plate with foci/plates scored	Mean no. of transformed foci/plate	TF ($\times 10^{-4}$)[a]
Untreated		4/6	1.17±0.48	7.45	2/6	0.53±0.25	5.52
DMSO (0.5%)		3/6	0.67±0.33	5.15	2/6	0.47±0.12	4.61
3-MCA	2.5	6/6	2.17±0.60[d]	20.67			
B(a)P	2.5				6/6[b]	3.17±0.48[e]	40.64
Captafol	0.1	6/6	5.00±0.58[e]	46.73	6/6[b]	4.50±0.72[e]	58.44
	0.05	5/6[b]	1.50±0.50	12.30	5/6[b]	2.50±0.76[e]	29.06
	0.01	2/6[c]	0.33±0.21	2.44	4/6[c]	2.33±0.80[d]	27.09
Captan	1	4/6[c]	1.33±0.56	12.55	6/6[b]	2.50±0.72[e]	25.77
	0.5	2/6[c]	0.33±0.21	3.00	6/6[b]	4.00±0.45[e]	49.39
	0.1	3/6[c]	0.50±0.22	4.00	6/6[b]	2.67±0.33[e]	33.38
Folpet	10	3/6[c]	0.50±0.22	0.00	6/6[b]	3.33±0.21[e]	32.02
	5	2/6[c]	0.33±0.21	3.11	6/6[b]	1.67±0.33[e]	21.69
	1	2/6[c]	0.50±0.34	4.39	4/6[c]	1.83±0.70[d]	20.33

a) Transformation frequency expressed as the number of transformed foci per number of cells at risk after chemical treatment.
b, c) Significantly different from control (DMSO-treated plates) using the Fisher Yates test of significance in 2×2 contingency tables: *b*) $P < 0.05$; *c*) $P < 0.01$.
d, e) Significantly different from control (DMSO-treated plates) using the distribution-free Mann-Whitney unpaired *t* test: *d*) $P < 0.05$; *e*) $P < 0.01$.

activity of the three fungicides is particularly apparent after S-9 mix-induced activation in level-II transformation cultures and is comparable to, or even higher, than that of positive control (cells treated with B(a)P) at all tested doses (Table IV). Although some spontaneous transformation was detectable in the negative controls (untreated cells and solvent-treated cells), which reflects the high sensitivity of the BALB/c 3T3 cells to the transforming activity of the chemicals,[25] the transformation activity exerted by the three pesticides, particularly in the level-II assays, is significantly high, as judged by the mean numbers of transformed foci and by the TF values, which were about 5- to 10-fold higher than the values of the negative controls. The relative cell-transforming potency, as ratio of net TF values by the single most effective dose, is: captafol > captan > folpet.

The data reported here are consistent with previous reports (see the introduction) indicating either genotoxic effects of these compounds in *in vitro* short-term assays or genotoxic and/or carcinogenic effects in *in vivo* experiments. However, some discrepancies exist in the literature on genotoxic and carcinogenic activities of the compounds *in vitro* and *in vivo*. It is likely that the reason for these discrepancies is the employment of different experimental models and/or the choice of the parameters studied. Such differences could also help to explain the discrepancy between the S-9 mix-elicited cell-transforming ability of the three pesticides seen in the present study

and the S-9-induced inhibition of genotoxic activity on prokaryotic cells observed in previous studies.[3, 29] Metabolism of captan is similar to that of captafol and of folpet due to their similarity in molecular structure, and involves a number of different and complex pathways.[28] In particular, the different metabolic pathways are correlated to the presence of thiols, e.g. cysteine and glutathione, and can generate either non genotoxic compounds or metabolites capable of inducing DNA adducts.[28, 30] In our experimental model, metabolism of the compounds could be affected by their conjugation with glutathione present in the cytosolic fraction of S-9 and by the presence of thiols in the serum added to the culture medium. Conjugation with glutathione seems to be confirmed by the inhibition of the cytotoxicity of these compounds following exposure to S-9-containing cultures (Table II). This metabolic pathway is, in fact, the most powerful in inducing detoxification of exogenous compounds. However, it cannot be excluded that S-9 could also induce the formation of other metabolites. According to Schechtman, further metabolites may originate from the endogenous metabolism of BALB/c 3T3 cells induced by S-9 itself.[31] The addition of S-9 mix may also induce some degree of variability in the cell transformation rate, depending on the S-9/substrate ratio.[31] This variability increases in the level-II amplification test where cells from randomly chosen level-I plates are replated.[27] Thus, not every level-I plate may contain

incipient transformed cells able to give detectable transformed foci, consequently affecting the apparent dose-response relationship.

The *in vitro* experimental model utilized in this study is a medium-term assay which is more useful than short-term assays to evaluate possible activities of chemicals in inducing carcinogenesis. In fact, BALB/c 3T3 cells, which have reached an indefinite lifespan (immortalization),[32, 33] have already undergone the first step(s) of the process of carcinogenesis involving genomic alterations. It is likely that xenobiotics act in transformation of these cells by causing further molecular changes possibly involved in the process of carcinogenesis.

Captan, captafol and folpet showed carcinogenic potential in medium-term *in vivo* assays in which the experimental animals were treated with these pesticides just after treatment with powerful carcinogens.[16, 17] These results and those from the present study demonstrate that *in vivo* and *in vitro* medium-term assays may be useful to assess the oncogenic potential of chemical compounds for humans. These procedures are more reliable than short-term mutagenicity assays; they allow the detection of chemicals acting as non mutagenic agents (carcinogenesis promoters), and are probably candidates to replace the usual long-term assays in rodents in the near future. Finally, it is interesting to note that captafol, whose utilization, unlike that of captan and folpet, has been prohibited in Italy since 1990, proved to be the most powerful cell-transforming agent in this study.

ACKNOWLEDGMENTS

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