The mutagenic effects of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine in Muta^{TM}Mouse colon is attenuated by resveratrol

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Abstract

Epidemiology studies show that consumption of certain naturally occurring chemicals (generally plant-derived) can protect against the development of cancer (chemoprevention). Resveratrol, a phytoalexin found in foods such as grapes and wine as a glucoside , is one such chemical. Our group has previously shown that treating mammalian cells with resveratrol aglycone can reduce the mutagenicity of the cooked meat-derived carcinogens 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). However, the anti-mutagenic effect of resveratrol *in vivo* has not been previously reported. In this study, daily treatment of resveratrol up to 1000μg/kg for 10 days was well tolerated in Muta^{TM}Mouse mice. Treating Muta^{TM}Mouse mice with the meat carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine induced mutation in the colon. Co-treatment of resveratrol and PhIP reduced the mutation frequency induced by PhIP in the colon by about 25% in a treatment group of mixed male and females. Analysing males and females separately revealed a sex difference in the response to PhIP and to the effect of resveratrol on PhIP-induced mutagenicity. In males compared to females, PhIP was a more potent colonic mutagen and resveratrol was more effective at attenuating the mutagenic response (~35% in males but only 9% in females). The reason for this sex difference in both PhIP mutagenicity and response to resveratrol is not clear. However, resveratrol treatment was also shown to powerfully inhibit ethoxyresorufin-O-deethylase activity in vivo, indicating that the antimutagenic affects are likely linked to metabolic activation of PhIP. These data suggest that resveratrol has anti-mutagenic effects *in vivo,* supporting its potential to act as a chemopreventative.

Introduction

There is increasing interest in trying to prevent cancer through the administration (usually oral) of a chemical or chemicals that are found in the natural environment (cancer chemoprevention) [1] ; [2]. Resveratrol, a phytoalexin found in many human consumables such as grapes and red wine [3] shows promise as an agent of this type.

A number of heterocyclic amines (HCAs) are formed during the routine cooking of red meat and meat containing products [4], therefore populations who consume high levels of red meat are potentially exposed to HCAs. To date, all of the food-derived HCAs that have been examined have been shown to be carcinogenic in rodents [5]. In humans, the association between red meat intake and cancer, specifically cancer of the breast and colon, is well established and has been reported in many food-related case-control studies [6]; [7]; [8]. It has been shown that metabolic activation of the food-derived HCA pro-mutagens is *via* the oxidation of the exocyclic amine group by cytochrome P450 (CYP) 1A family enzymes [9]; [10]; [11]; [12]. This is followed by subsequent phase II metabolism, by enzymes such as N-acetyltransferase [13]. Both of these metabolic steps are carried out with greater efficiency in human hepatic tissue than that of mice and rats [14].

The most abundant HCA formed in cooked beef is PhIP[15], thus humans are exposed to higher levels of this than any other HCA. PhIP is mutagenic and has been shown to induce mutations in both the *lacZ* transgene of Muta[TM]Mouse [16] and the *lacI* transgene from the intestine of BigBlue mice [17]. Of the several types of mutation found in these models, GC→TA transversions were the most abundant and a significant number of -1 guanine frameshifts were also noted. Many of these mutations occurred at 5'-GGA-3' motifs. This pattern of mutation is very similar to that found *in vitro* [18].

It has previously been demonstrated by our group and others that resveratrol aglycone can inhibit chemical-induced mutation in both bacterial and mammalian cells [19]; [20]. Whether

resveratrol can exert similar activity *in vivo* is not clear, however, studies suggest there are potential beneficial effects of resveratrol consumption. For example it has been shown that 0.01% resveratrol aglycone in the drinking water of *Apc^min* mice (genetically predisposed to develop intestinal tumours) for 7 weeks decreased the formation of tumours in the small and large intestine by up to 70% [21], while in another study, administration of resveratrol aglycone (200 µg/kg/day) in drinking water to azoxymethane-treated male F344 rats for 100 days decreased the formation of aberrant crypt foci in the colon [22]. These studies therefore suggest that resveratrol has anti-mutagenic and anti-carcinogenic potential *in vivo*.

In the current study, the mouse model Muta™Mouse was used to test the hypothesis that resveratrol aglycone has anti-mutagenic effects *in vivo*, using the food-borne carcinogen PhIP as a model food-derived genotoxicant. The Muta™Mouse model employs a bacterial *LacZ* gene as a reporter for mutation. These reporter genes have been integrated into the mouse chromosome, which can be recovered as phage particles. We report that treatment of Muta™Mouse with resveratrol aglycone attenuates the colonic mutagenicity of PhIP and that there is a pronounced sex difference in both the mutagenic response to PhIP and its inhibition by resveratrol.

Material and Methods

Chemicals

2-Amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP, >99% purity) was purchased from Toronto Research Chemicals Inc (Toronto Canada). Ethylnitrosourea (ENU, >99% purity), trans resveratrol aglycone (>99% purity), Luria Bertani broth (LB broth), kanamycin, ampicillin, phenyl β-D galactosidase (P-Gal) and 5-bromo-4-chloro-3-indolyl β-D-galactose (X-Gal) were purchased from Sigma Chemical Co. (Poole, UK). The RecoverEase™DNA

isolation kit, RNAce ribonucleotide cocktail, proteinase K, Transpak packing extract were all purchased from Stratagene (La Jolla, California).

Animal Husbandry

Male CDF1 and male and female Muta^{TM}Mouse mice (6-8 weeks old) weighing 20-40 g were obtained from Covance (Harrogate, UK); bottled (public supply) water and diet was provided *ad libitum*. All animals were treated according to the Animals (Scientific Procedures) Act (1986), housed in a temperature and humidity controlled environment (20±1ºC) with 12 h of light and 12 h of darkness, and after any treatment, were checked twice daily for any signs of toxicity.

Mutation studies using the mouse model Muta^{TM}Mouse

Animals were weighed before dosing, and the volume of ENU positive control, PhIP, resveratrol aglycone or vehicle negative control was calculated based on the daily weights. Six animals (3 male and 3 female) were dosed in each of the five treatment groups. Male and female Muta^{TM}Mouse mice were treated as detailed in Table 1. ENU was prepared in sterile purified water and all PhIP and resveratrol doses were prepared in DMSO: H$_2$O (1:1 by volume). All treatments were administered at 10ml/kg body weight. After treatment, a mutation expression period of 20 days was allowed before killing the animals by a schedule 1 method.

DNA isolation from the colon and liver of treated Muta^{TM}Mouse mice

Large molecular weight DNA from the colon and the liver was recovered using the Stratagene RecoverEase^{TM} DNA isolation kit. Briefly, the tissue was washed in ice cold saline and 50–200 mg of complete tissue was homogenised in 8ml ice-cold lysis buffer using

a 7ml Wheaton Dounce tissue grinder. The homogenate was poured through a sterile cell strainer and centrifuged at 1100xg for 12 min at 4°C. The supernatant was discarded and 50μl digestion solution (20μl RNAce ribonucleotide cocktail/ml buffer) was added to the remaining pellet. Immediately, 50μl of pre-heated (50°C) proteinase K solution was added and the final reaction mixture was incubated for 45 min at 50°C. After the incubation the reaction mixture was placed in a dialysis cup and dialysed against TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA; 0.5 L/ tissue sample) at room temperature for 24-48h. Following dialysis, hydrated total genomic DNA was recovered and stored at -20°C until required.

Bacterial cell culture

A culture of the bacterial strain *E.Coli* (ΔLacZ, Gal E⁻,recA⁻,Kan^r (gal E⁻,Amp^r)) [23], prepared from a single colony on a freshly streaked plate, was grown overnight in an orbital incubator at 37°C, 200rpm, with LB broth (20g LB mix dissolved in 1L deionised water, sterilised by autoclaving), 0.2% maltose, 0.1mg/ml ampicillin and 0.02mg/ml Kanamycin. The following morning a 'day culture' was prepared from 1-2% of the 'overnight culture' in fresh LB broth and 0.2% maltose. After the bacterial cells had reached an OD_{600} 1.0, they were pelleted and resuspended in fresh LB broth containing 10mM $MgSO_4$ to give an OD_{600} 2.0. The cells were kept on ice until needed.

DNA packing and mutant selection

Shuttle vectors (λgt10*lacZ*) were rescued from the genomic DNA of the liver and colon and single copies packaged into bacterial phage particles, using Transpak (Stratagene) packing extract following the manufacturer's instructions. One vial of Transpak packing extract (10μl) was used for each DNA sample. The extract was kept on dry ice (-40°C) until needed. The contents of the tube were quickly thawed before use. Using a wide-bore pipette

tip, 5-10µl of genomic DNA (0.5-1mg/ml) was added to the thawed Transpack packing extract. The DNA- Transpack packing extract mixture was then incubated at 30°C for 90 min. After this, a further 12µl of Transpack packing extract was added to each sample and incubated for a further 90 min at 30°C. After this, 500µl of sterile SM buffer (1M Tris.HCL pH 7.5, 10mM MgSO₄, 100mM NaCl, 0.01% gelatin) was added to each packaging reaction and vortexed.

The packaged phage were used to infect a culture of *E. Coli* (ΔLacZ,GalE⁻,recA⁻ ,Kanʳ(galE⁻,Ampʳ)) [23], which were plated out under non-selection and selection conditions. Selection was achieved by plating phage-absorbed bacteria onto agar containing the lactose analogue phenyl β-D galactoside (P-Gal). Under non-selection conditions (no P-Gal), all phage-infected bacteria form colonies, giving a count of the total number of packaged *LacZ* genes. Confirmation of mutant *LacZ* genes was accomplished by "picking" individual mutant plaques, re-suspended in SM buffer, vortexed vigorously and an aliquot 10-15 µl used to infect a strain of *E.coli* (*LacZ*). In the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), mutant *LacZ* infected bacteria produced clear or light blue viral plaques. Non-mutants produced dark blue plaques. The mutation frequency for each sample of DNA was calculated as described by Dean and Myhr [24].

Ethoxyresorufin O-demethylase activity.

CD2F1 mice were given resveratrol (10, 100, 1000 µg/kg in 50 µl DMSO/H₂O 1:1 v/v) by oral gavage (po). DMSO:water vehicle (1:1/50 µl) was used as a vehicle control. Half of the animals were dosed once a day until day five, and killed by cervical dislocation 24h after the last dose. The remaining animals continued to be dosed until day ten and killed on day eleven. The liver was removed from each animal, visually examined, weighed and hepatic microsomes prepared and stored as previously described[9]. The ethoxyresorufin O-

deethylase (EROD) assay was used to determine CYP1A activity[11]. The formation of fluorescent resorufin was measured using a Shimadzu RF 540 Spectrofluorimeter with excitation at 535nm and emission at 585nm. Under these conditions, formation of resorufin is linear for at least 15 min and the fluorescence is proportional to the metabolic activity of the CYP enzymes.

Statistical analysis

Statistical analysis was performed using ANOVA with Tukey or Dunnet's post-test.

Results

The effect of resveratrol on PhIP-induced mutation in Muta^{TM}Mouse

The transgenic mouse model, Muta^{TM}Mouse, was used to assess the anti-mutagenic activity of resveratrol following co-treatment with the food carcinogen PhIP. To check for overt signs of treatment related toxicity, observations were made on all animals prior to dosing and 0.5h and 2h after dosing. No distress was noted to any animal and every treatment was well tolerated. The colon and liver from 6 animals (3 male and 3 female) from each treatment group were analysed for mutation frequency (MF) at the LacZ transgene (Tables 2 and 3).

The colon of ENU treated positive control mice contained a large number of mutated transgenes (1646 ± 350 and 1047 ± 168 ×10^{-6} (mean ± SD) plaque forming units (pfu) for male and female, respectively) (Table 2). In contrast the DMSO:H$_2$O negative control had a much lower colonic mutation frequency (127 ± 20 and 90 ± 436 (mean ± SD) plaque forming units (pfu) for male and female respectively), (Table 2). ENU was also mutagenic to the liver, (321 ± 95 and 297 ± 177 x10^{-6} (mean ± SD) plaque forming units (pfu) for male and female, respectively), but much reduced compared to the colonic mutation frequency (Tables 2 and

3). Similarly, the DMSO:H_2O negative control also had a reduced hepatic mutation frequency (70 ± 12 and 46 ± 4 (mean ± SD) plaque forming units (pfu) for male and female respectively), (Table 3). Thus for both male and females, ENU was a much less potent mutagen in the liver compared to the colon.

Resveratrol (1mg/kg) administration did not significantly alter the MF in the colon (125 ± 22 and 84 ± 26 ×10^{-6} pfu for male and female, respectively) or in the liver when compared to the DMSO:H_2O vehicle control (Tables 2 and 3).

Treatment with 20 mg/kg PhIP significantly (p<0.001) increased the MF in the colon both in male and female mice compared to the vehicle control group (Table 2). Moreover, the MF induced in the colon was 42.6% lower in female compared to male PhIP-treated mice (Table 2). Co-treatment of resveratrol with PhIP significantly reduced the PhIP-induced MF in the colon by ~35% in males (P<0.001; Table 2), however, the same comparison in females showed only a reduction of approximately 9% (Table 2). In the liver of treated males, the PhIP mutation frequency was elevated compared to the male vehicle controls, but this was not statistically significant and there was no effect of resveratrol on hepatic PhIP mutation frequency (Table 3). In females, neither PhIP, nor resveratrol nor PhIP plus resveratrol had any statistically significant effect on the hepatic mutation frequency (Table 3).

Effect of resveratrol treatment on Ethoxyresorufin O-demethylase activity.

CD2F1 mice (genetically similar to Muta^{TM}Mouse) that were treated daily with up to 1000μg/kg resveratrol for 5 days (group 1) or 10 days (group 2) did not show any overt signs of stress and the compound did not induce mortality or morbidity during the experimental period. Macroscopic observation of gastrointestinal and hepatic tissue did not reveal any gross sign of inflammation or abnormality in any treatment group. The average weight of each mouse in all treatment groups (1:1 DMSO:H2O control, 10 μg/kg resveratrol, 100 μg/kg

resveratrol, 1000 µg/ kg resveratrol) was not significantly different before or after treatment (data not shown).

The effect of resveratrol treatment on hepatic microsomal EROD activity is presented in Figure 1. EROD activity was inhibited by greater than 50% following 5 days treatment with 1000 µg/kg resveratrol, and more than 40% and 95% after 10 days treatment with 100 and 1000 µg/kg respectively, when compared to the DMSO:H_2O control. Indeed, the fact that EROD activity is almost completely lost after 10 days treatment with 1000 µg/kg resveratrol is remarkable in view of the lack of any signs of liver toxicity in the mice. The dose-dependent decrease in EROD (CYP1A activity) supports the suggestion that resveratrol or its metabolites inhibit CYP1A activity *in vivo*.

Discussion

Transgenic mouse models containing recoverable bacterial reporter genes for measurement of *in vivo* mutation, such as Muta[TM]Mouse, are helpful for identifying mutagenic chemicals. In the current study, treatment of male and female Muta[TM]Mouse mice with resveratrol (1 mg/kg, once daily for 10 days) was well tolerated and did not cause any outward distress to either sex of animal. Furthermore, despite the reputed ability of resveratrol to accumulate within selected tissues in the body, especially the liver [25], there was no evidence of macro-toxicity to the mice after dissection and macroscopic observation of the tissues (gastrointestinal tract, liver, lungs, heart, and kidneys). These observations looked for tissue swelling, abnormal growths, ulcerated tissue and inflammation.

The heterocyclic amine PhIP has been shown to be mutagenic to several tissues in the transgenic mouse and rat models Muta[TM]Mouse and BigBlue [16; 17; 26; 27]. The data presented in the current study show PhIP to be a potent mouse colonic mutagen under the experimental conditions employed. This is in agreement with the study by Lynch *et al.* [16] who showed that

PhIP (20 mg/kg, 4 daily doses, seven day expression period) induced a significant 5.9 fold increase in colonic MF and only a very marginal effect in liver. Although the PhIP-induced MF observed in the colon in the current study was lower than that observed by Lynch *et al.* [16] it has previously been suggested that a longer exposure time to the HCA can increase the mutagenic effect in the *lacZ* and *lacI* transgenes [16; 17] and direct comparisons are difficult since the experimental protocols were different. It is important to note that the apparent tissue selectivity of PhIP-induced mutation (colon > liver) is consistent with PhIP's reported carcinogenicity; i.e. as an intestinal rather than hepatic carcinogen [14].

We have previously demonstrated the anti-mutagenic effect of resveratrol following PhIP treatment *in vitro* [20] and the current study shows that this effect also occurs *in vivo* in Muta™Mouse. There have been no reports to date suggesting that resveratrol itself is mutagenic in the colon, and the data presented here confirm this.

When male and female mice were analysed separately it was observed that PhIP induced approximately 50% more mutation in the colon in males compared to females. A significant decrease in colon MF (~35% reduction) was observed for male mice following co-treatment with resveratrol and PhIP compared to PhIP alone, but the effect was much less in females (9% reduction). It is also worth noting that treatment with ENU induced a higher MF in male animals compared to females. Consistent with the present study, difference between the genders in the mutagenic potential of ENU and the heterocyclic amines have been previously reported [16; 28; 29 30]. The reason for this difference is not known, but is likely to be multifactorial and include differences in metabolism, (rate of activation versus rate of deactivation), toxicokinetics and the ability to repair DNA damage. It is also noteworthy that PhIP like resveratrol has been shown to act as a potent oestrogen [31] and as such might be expected to affect the genders differently. The potential of this latter activity to affect mutation and cancer has previously been reported [32].

In the current study, 1 mg/kg resveratrol, the dose used to treat the mice *in vivo,* was found to be well tolerated with no obvious clinical signs of toxicity. This is consistent with a number of other *in vivo* rodent studies that observed biological effects at resveratrol concentrations equal to or less than this dose [22]. The doses of resveratrol employed here have been reported to be representative of concentrations that could occur through the consumption of red wine or red grapes [33]. It should be noted that the natural occurrence of resveratrol is as glycosides. Roches-Ribalta *et al* [34] have characterised the pharmacokinetics of resveratrol metabolites in humans after consumption of red wine and grape extract and report extensive metabolism with tissue and gut microbial involvement leading to a rapid appearance of plasma resveratrol glucuronide and sulphate conjugates.

It has been shown that multiple dosing of Muta™Mouse with PhIP is effective for genotoxicity[16] and in support of this, data presented here show that multiple daily doses of 20mg/kg PhIP produce an effective genotoxic response in the colon of Muta™Mouse. Resveratrol treatment did reduce PhIP-induced mutation in the colon of Muta™Mouse, significantly so in the colon of male animals, possibly by reducing bioactivation of PhIP through inhibition of CYP activity [20]. In support of this mechanism, we examined the effect of resveratrol treatment on the hepatic drug metabolism activity (EROD) of CD2F1 mice (genetically similar to Muta™Mouse). Resveratrol treatment (100 and 1000μg/kg) inhibited EROD activity (a marker for CYP1A activity), and this effect was potentiated following a longer exposure (10 days of resveratrol treatment). In support of this, *in vitro* studies have shown that resveratrol can inhibit CYP1A1 with low affinity (IC50=1.2mM) [35], which we have confirmed in CDF1 mouse microsomal preparations (data not shown). Clearly, attenuation of this enzyme activity could influence activation of PhIP, whose genotoxicity is CYP1A-mediated. In this context, it is important to note that our analysis of metabolic activity was determined 1 day after the last dose of resveratrol but analysis of mutagenic

effects was determined after an additional 20 days to achieve optimum expression of the transgene. Clearly this discrepancy means that direct comparisons are not possible, but may imply recovery of the CYP1A activity and therefore activation of residual PhIP, may have occurred in the Muta[TM]Mouse study.

The DNA-protective effect of resveratrol treatment, which has been observed in bacteria and mammalian cells [20]; [19], may not be entirely an inhibition of mutation *per se*, but more likely a combination of adduct-formation block and a decrease in cell growth *i.e.* an inability of the mutated phenotype to proliferate. Some of the reported biological actions of resveratrol such as induction of apoptosis or the inhibition of ribonucleotide reductase, DNA polymerase and the correct functioning of the cell cycle, confirm the multiple biological activities of the compound, all of which may contribute to a chemopreventive effect [3]; [22]. Moreover, PhIP like resveratrol has been shown to act as a potent oestrogen [31] and as such might be expected to affect the genders differently.

Our results show that resveratrol has anti-mutagenic properties *in vivo* in Muta™Mouse, which appears to be more potent in males than females, possibly through inhibition of CYP enzymes. These data support the proposal that resveratrol has potential as a chemopreventive agent.

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Conflict of Interest Statement

The authors declare there are no conflicts of interest.

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Tables

Table 1. Summary of the dosing schedule for each Muta^{TM}Mouse group.

Group	*Day of Study*									
Number	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	ENU
2	VEH	VEH	VEH	VEH	VEH	VEH	VEH	VEH	VEH	VEH
3	VEH	VEH	P	P	P	P	P	P	VEH	VEH
4	R	R	R	R	R	R	R	R	R	R
5	R	R	R+P	R+P	R+P	R+P	R+P	R+P	R	R

ENU   : ethylnitrosourea 100mg/ kg (positive control) – ip in water
VEH   : Vehicle (50:50 DMSO & water mix) – po
P   : PhIP 20mg/ kg – po in DMSO/ H_2O (1/ 1)
R   : 1mg/ kg resveratrol - po in DMSO/ H_2O (1/ 1)
R+P   : 1mg/ kg resveratrol + PhIP 20mg/ kg – po in DMSO/ H2O (1/ 1)

All animals were killed and necropsied on day 30

Table 2. The effect of treating MutaTMMouse mice with PhIP and resveratrol on individual mouse colonic mutation frequency.

Treatment	sex[a]	PFU[b]	Mutants	MF[c]	MF Mean ± SD
ENU (100mg/kg)	M	401,440	753	1878	1646 ± 350
	M	852,800	1549	1817	
	M	587,080	595	1243	
	F	1,013,480	1027	1014	1047 ± 168
	F	577,800	519	898	
	F	652,440	802	1230	
PhIP (20 mg/kg)	M	642,160	409	637	580 ± 51[+§]
	M	824,720	442	536	
	M	548,600	311	567	
	F	539,760	193	358	333 ± 77[§]
	F	574,340	142	247	
	F	637,440	251	395	
PhIP (20 mg/kg) + Resveratrol (1 mg/kg)	M	39,080	15	383	388 ± 23*[+]
	M	495,040	182	368	
	M	616,940	255	414	
	F	672,160	187	279	302 ± 26
	F	446,680	132	296	
	F	487,800	161	331	
Resveratrol (1 mg/kg)	M	499,200	73	147	125 ± 22
	M	554,320	70	127	
	M	681,720	69	102	
	F	343,120	39	113	84 ± 26
	F	727,480	44	60	
	F	54,080	4	80	
Vehicle control	M	635,960	95	150	127 ± 20
	M	458,640	51	112	
	M	704,600	84	120	
	F	572,000	72	126	90 ± 43
	F	3,111,680	130	42	
	F	197,600	20	103	

[a]M= Male animals, F= Female animals.
[c]Mutation frequency (MF) is expressed per 10^6 plaque-forming units (pfu) b.
* Significantly different from PhIP treatment ($p < 0.001$) ANOVA with Tukey post test
[+] Significantly different from same treatment female ($p < 0.001$)
[§] Significantly different from vehicle control (DMSO/H_2O; $p < 0.001$)

Table 3. The effect of treating MutaTMMouse mice with PhIP and resveratrol on individual mouse hepatic mutation frequency.

Treatment	sex[a]	PFU[b]	Mutants	MF[c]	MF Mean ± SD
ENU (100mg/kg)	M	650,280	151	233	321 ± 95[§]
	M	637,480	269	422	
	M	519,280	160	309	
	F	770,400	144	187	297 ± 177[§]
	F	24,440	5	203	
	F	354,120	177	502	
PhIP (20 mg/kg)	M	557,400	40	73	130 ± 56
	M	635,720	118	185	
	M	354,120	46	131	
	F	370,960	38	102	77 ± 31
	F	518,440	23	43	
	F	444,200	38	87	
PhIP (20 mg/kg) + Resveratrol (1 mg/kg)	M	424,440	22	52	84 ± 35
	M	580,840	71	121	
	M	656,760	52	78	
	F	577,000	41	71	56 ± 13
	F	411,480	30	50	
	F	245,960	12	48	
Resveratrol (1 mg/kg)	M	518,440	59	113	75 ± 37
	M	275,440	20	73	
	M	855,520	35	40	
	F	657,480	60	91	75 ± 14
	F	306,800	20	63	
	F	35,720	3	70	
Vehicle control	M	257,400	19	72	70 ± 12
	M	535,600	31	57	
	M	659,360	54	81	
	F	884,000	43	50	46 ± 4
	F	368,160	17	46	
	F	208,520	9	42	

[a]M= Male animals, F= Female animals.
[c]Mutation frequency (MF) is expressed per 10^6 plaque-forming units (pfu) b.
[§] Significantly different from vehicle control (DMSO/H2O; p<0.001) ANOVA with Tukey post test
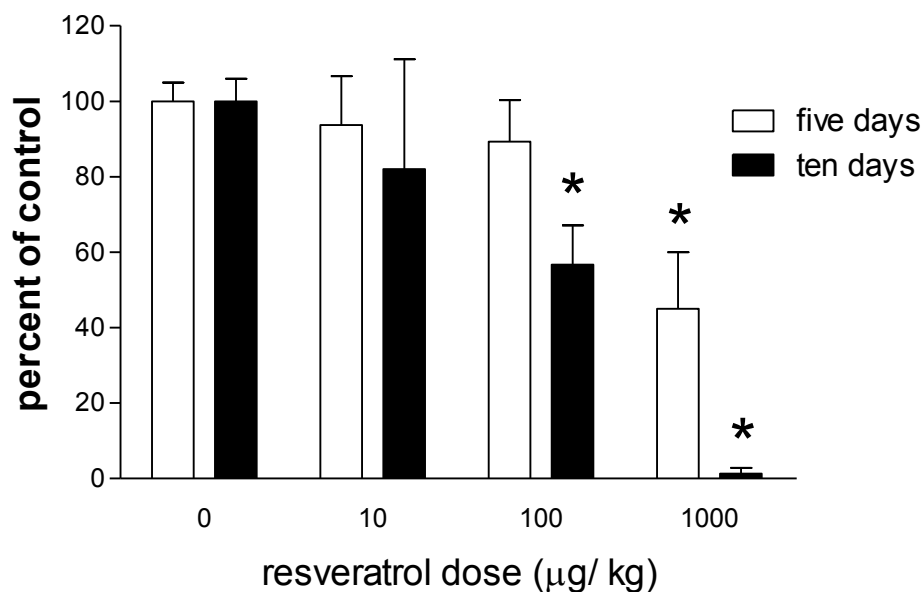
Figure 1. The ethoxyresorufin O-deethylase activity (EROD) of hepatic microsomes isolated from CD2F1 male mice treated with resveratrol for 5 (□) and 10 (■) days.

Resveratrol was prepared in DMSO:H_2O (1:1 by volume) vehicle. All treatments were given once a day by oral gavage at the doses indicated. Animals were killed 24 h after the last dose.

Results are the mean ± SD of 5 individual mice per dose. All assays were performed in triplicate and were analysed for statistical significance. * P<0.01 compared to respective DMSO control (one way-ANOVA with Dunnett's post test).