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The Molecular Mechanism of Safrole-induced DNA Adducts and its Role to Oral Carcinogenesis

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IARC classified areca quid as a human carcinogen. Areca quid chewed in Taiwan includes *Piper betle* inflorescence, which contains high concentrations of safrole (15 mg/fresh weight). Safrole is a documented rodent hepatocarcinogen, and chewing areca quid may contribute to human exposure (420 μm in saliva). The carcinogenicity of safrole is mediated through 1'-hydroxysafrole formation, followed by sulfonation to an unstable sulfate that reacts to form DNA adducts. Using human liver microsomes and *Escherichia coli* membranes expressing bicistronic human P450s, CYP2E1 and CYP2C9 were identified as the main P450s involved in the activation of safrole.

We have demonstrated the presence of stable safrole-dGMP adducts in human oral tissues following areca quid chewing using ^{32}P-postlabeling and HPLC mass spectrometry methods. By studying 88 subjects with a known AQ chewing history and 161 matched controls, we have demonstrated that the presence of safrole-DNA adducts in peripheral blood cells was correlated to AQ chewing, and CYP2E1 seemed to play an important role in the modulation of safrole-DNA adduct formation. We have also shown that safrole can form stable safrole-DNA adducts as well as oxidative damages in rodent liver. However, the stable safrole-DNA adducts may represent a more significant initial lesion as compared to the rapidly repaired safrole-induced 8-hydroxy-2'-deoxyguanosine. This oxidative DNA damage is mediated through the formation of hydoryxchavicol, the major safrole metabolite in human urine. Hydroxychavicol may have gone through two-electron oxidation to the o-quinone; then via one-electron reduction to semiquinone radicals to generate oxidative DNA damage. However, these reactive metabolites can be efficiently conjugated by GSH. These data suggest that safrole may contribute to the initiation of oral carcinogenesis through safrole-DNA adduct and not oxidative DNA damage. In addition, CYP2E1 may modulate this adduct formation.

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Introduction

- There is sufficient evidence that *chewing betel quid (AQ) containing tobacco* is carcinogenic to humans. (IARC, 1985).
- There is inadequate evidence that *chewing BQ without tobacco* is carcinogenic to humans. (IARC, 1985).
- Betel quid and areca nut are carcinogenic without tobacco (Lancet Oncol. 2003 4(10):587)
- The component of BQ varies in different geographical locations.
- BQ may have different carcinogenic potentials.

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Piper betle inflorescence

- Used in Taiwan, part of Guam and Papua New Genia.
- PBI contains 15 mg/g safrole
- Chewing BQ may contribute to safrole exposure (420 μM in saliva).

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Safrole

- an essential oil, present in many herbs
- Genotoxicity, conflict results in different *in vitro* test systems
- a "weak hepatocarcinogen" by IARC
- ≥0.5% in diet, lead to hepatoma
- <0.5% in diet, no cancer, in the 2-yr test period
- Induces stable hepatic safrole-DNA adducts
- Whether safrole induces oxidative damage *in vivo* has not been documented.

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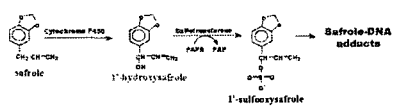

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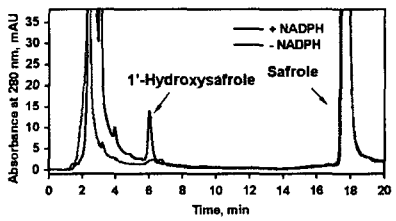
The HPLC chromatography of safrole 1'-hydroxylation assay of human liver microsomes



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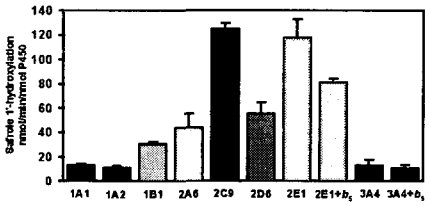
Safrole 1'-hydroxylation by *E. coli* membranes expressing bicistronic human cytochrome P450 enzymes



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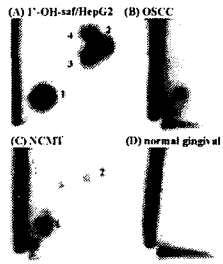

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Safrole-DNA adduct	BQ chewing[a]					
	Yes			No		
	OSCC (n=30)	NCMT (n=30)	OSF (n=7)	OSCC (n=6)	NCMT (n=6)	Normal gingiva (n=14)
Present	23	29	6	0	0	0
Absent	7	1	1	6	6	14
Levels[b]						
Mean± SE	4.0± 0.9	9.7± 2.7	7.8± 1.5			
Median[c]	2.2	5.0	9.1			
Range	0–19.4	0–65.3	0–11.7			

[a]P<0.001 by Fisher's exact test, for BQ chewer *v.s.* non-BQ chewer.
[b]Adduct levels are express as adducts/10^8 nucleotides.
[c]P<0.05 by Mann-Whitney test, for OSCC *v. s.* NCMT or OSF

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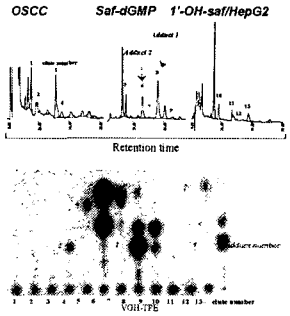

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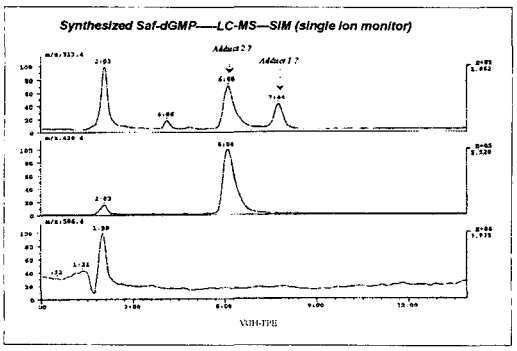

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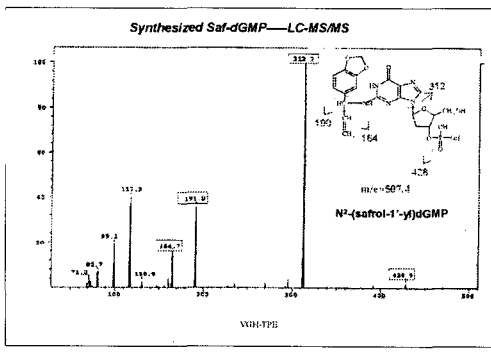

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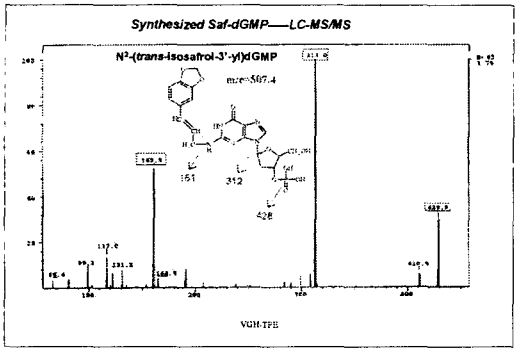

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Conclusion

- We have found safrole-DNA adducts in OSCC (23/30) and matched NCMT (29/30).
- This adduct was not detectable in non-BQ related OSCC and normal gingival tissue ($p<0.001$).
- This adduct level in OSF and NCMT was significant higher ($p<0.05$) than in OSCC.
- This adduct was identical to synthetic safrole-dGMP adducts.
- This suggest that safrole may contribute to oral carcinogenesis.

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Table 1
Detection of safrole-DNA adducts in AQ chewers and controls

	Areca quid chewers ($N = 88$)	Controls ($N = 161$)
Age (mean ± S.D.)	50.07 ± 8.86	50.35 ± 8.28
Safrole-DNA adduct		
Positive[a] (%)	83 (94.32)	21 (13.04)
Negative (%)	5 (5.68)	140 (86.96)
RAL (adducts/10^8 nucleotides)		
Mean ± S.D.	6.09 ± 8.75	0.21 ± 0.80
Median	3.08	0
Range[b]	0–70.62	0–8.13

RAL, relative adduct labeling; S.D., standard deviation.
[a] $P < 0.00001$ by chi-square test.
[b] $P < 0.00001$ by Mann Whitney test.

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Table 4
Combined effects of the CYP2E1 and GSTM1 genotypes on safrole-DNA adduct formation among AQ chewers chewing less than 20 betel quids per day

Genotypes		Safrole-DNA adduct levels[a]		OR (95% CI)[b]	Adjusted OR[c] (95% CI)
CYP2E1	GSTM1	RAL < 25 (*)	RAL ≥ 25 (*)		
Group I					
c1c1	Null	9 (47.4%)	10 (52.6%)	1	1
Group II					
c1c2+c2c2	Null	9 (19.1%)	38 (80.9%)	3.70 (1.16–11.79)	3.89 (1.03–11.81)[d]
c1c1	Null				

[a] Adduct levels are expressed as adducts/10^8 nucleotides.
[b] Multiple logistic regression model was used to adjust age, status of cigarette smoking and alcohol drinking.
[c] $P = 0.02$.
[d] $P = 0.046$.

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Time-dependent changes of 8-OH-dG in the liver of control and safrole-treated rats

	8-OH-dG/10^5 dG		
Safrole (mg/kg)	0	500	1000
3 days	3.48 ±0.20	4.12 ±0.25	5.09 ±0.46*
5	3.41 ±0.17	5.58 ±0.15*	7.18 ±0.34*
10	3.17 ±0.50	4.57 ±0.55	6.33 ±0.62*
15	3.88 ±0.42	4.72 ±0.53	4.47 ±0.28

* $p<0.05$ as compared with control using ANOVA with Dunnett's test

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Conclusion/Discussion

- Safrole dose-dependently induced oxidative damage *in vivo*, as evidenced by the elevation of hepatic LHP and 8-OH-dG.
- Oxidative damages can be blocked by the GSH-mediated detoxification systems.
- LHP peaked on day 3 and returned to basal level on day 15.
- On the other hand, 8-OH-dG peaked on day 5, and returned to basal level on day 15
- In contrast, safrole-DNA adducts can be detected in liver at 30 and 140 days after a low (1μg/mouse) and high (10 mg/mouse) dose administration, respectively.

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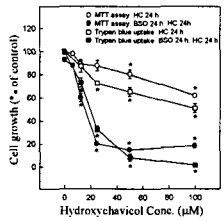

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- This study suggest that HC may have gone through 2e⁻ oxidation to the o-quinone; then via 1e⁻ reduction to semiquinone radicals to generate oxidative DNA damage and finally induction of cytotoxicity and apoptosis in GSH-depleted cells.
- The formation of HC-QM has not been confirmed in this study.
- HC may have limited cytotoxic potential in GSH competent cells.

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