COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REQUEST FROM ACNFP FOR ADVICE ON THE NOVEL FOOD INGREDIENT CYCLOASTROGENOL TA-65

Introduction

1. In the EU, a novel food is defined as one which does not have a significant history of consumption within the EU prior to May 1997. Such foods are subject to a pre-market safety assessment before a decision is made on an EU wide authorisation. An application is made to a Member State which reviews the application before giving an initial opinion. The opinion is then circulated to the other EU Member states for comment.

2. The Advisory Committee on Novel Foods and Processes (ACNFP) is a non-statutory, independent body of scientific experts that advises the Food Standards Agency on matters relating to novel foods and novel processes; this includes considering novel food applications.

3. The ACNFP asked COC to comment on aspects of a novel food application related to carcinogenicity for cycloastragenol TA-65[1], a compound extracted from plants intended for use in food supplements. The applicant states that the product is to be used for maintenance or enhancement of physiological functions within the body that are associated with well-being and health. However, it should be noted that the pre-market assessment does not consider efficacy or health claims.

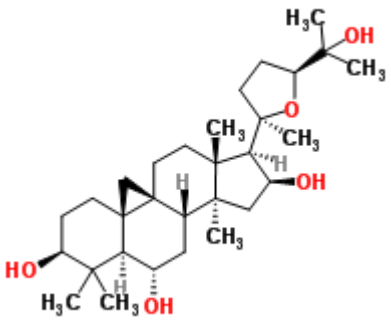
4. The COC considered the information and expressed a number of concerns regarding the available data and the appropriateness of the application given the possible mechanism of action of the compound. The COC concluded that the available data were inadequate to demonstrate a lack of carcinogenic potential and that a bioassay or other comparable work was needed. They also noted that genotoxicity data had been provided and while they noted the view of the manufacturers that the results were equivocal or negative, they recommended that the COM were consulted both to comment on the available data but also on whether the assays conducted were appropriate to demonstrate a lack of genotoxic potential. In particular, concerns were expressed about the notably the *in vitro* chromosome aberration study. In subsequent discussions with COM members, it was suggested that it might be worth looking at the effects of cycloastragenol in non- transformed cells (such as human lymphocytes) and that a pre-incubation Ames assay might be useful.

[1] Cycloastragenol is the aglycone of astragaloside IV. The novel food application is made in respect of the product cycloastragenol-TA65 (TA65) which is >98% cycloastragenol. Some of the experiments reported have used differing formulations of cycloastragenol and this has been noted in the text where applicable.

Background

5. Cycloastragenol is a compound isolated from the plant *Astragalus trojanus*. The plants contain *Astragalosides* which are the source of cycloastragenol-TA65. Cycloastragenol is a triterpene aglycone of the 9,19-cyclolanostane type and is one of the secondary metabolites found in all *Astragalus* species. The triterpene glycosides belong to the class of secondary plant metabolites known as saponins.

Fig 1. Structure of cycloastragenol



6. *Astragalus* species have been used as ingredients in traditional Chinese and Asian medicines. In particular, *Astragalus,* specifically the dried root of *Astragalus membranaceus* is used primarily as a tonic, particularly for the spleen and lungs.

7. The molecule was identified via an empirical screen (TRAP- the Telomere Repeat Amplification Protocol) based on its ability to upregulate telomerase.

History of the application

8. The application for the placing on the market of cycloastragenol TA-65 was considered by the ACNFP in 2014. The application consisted of data on the specification, production method, ADME, toxicology and microbiology. The publicly available section of the submission is attached at Annex 1. Key data from the submission are summarised below:

Absorption, Metabolism Distribution and Excretion

9. The intestinal absorption of cycloastragenol was studied using a Caco-2 cell monolayer model. The data in the submission are taken from a publication by Zhu *et al*., (2010) which reports work funded by the applicant. The compound investigated was described as TAT2 (a cycloastragenol of 90-95% purity generated by acid hydrolysis of astragalaside IV). The paper by Zhu *et al* (2010) is attached at Annex 2.

10. In the submission it was reported that because passage through the Caco-2 cell monolayer proceeded by passive diffusion with “minimal” metabolism, (stating that only 2 oxidised metabolites and four glucuronide conjugates were identified in the apical and basolateral sides of the monolayer) the possibility of rapid first pass

metabolism through the intestine was suggested. The metabolites were analysed by mass spectrometry (MS). Approximately 9% of the applied TAT2 reached the receiver side intact within 10 minutes, increasing to 38% after 60 minutes exposure, the rate of transport slowing after 60 minutes. Structural analysis of the conjugates was made based on MS fragment ions. However, the major identifiable fragments were those losing H_2O molecules and glucuronic acid from the original metabolites. The positions at which glucuronic acid was attached to cycloastragenol could not be determined with mass spectra only. The amounts of the metabolites obtained were too low to analyse by other means. Similarly, although two oxidised metabolites were identified it was not possible to determine their structure.

11. The time-dependent formation and excretion of the six cycloastragenol metabolites in the Caco-2 culture was monitored. Formation of the metabolites was slow, with 87% of the cycloastragenol remaining intact after 6 hours incubation.

12. In further experiments, extensive hepatic metabolism was suggested by incubating the cycloastragenol with rat and human microsomes; MS analysis indicated that only 17.4% and 8.2% of the parent compound was left after 30 minutes incubation with the rat and human microsomes respectively. As many as seven different metabolites were identified in the microsome samples. The metabolites were largely monohydroxylated with additional hydroxylation occurring post oxidation. The possible metabolites were suggested based on changes to molecular weights in the limited structural information that was available from the MS fragmentation. Zhu *et al*., (2010) note that hydroxylation and oxidation in the carbon ring and side chain are well known metabolic pathways for triterpene and steroid structures. The proposed fragmentation pathway and the mass spectrum are given in Figure 2 opposite.

13. The MS profile of metabolites produced by rat and human microsomes was qualitatively similar but quantitatively different. In humans, the predominant metabolite was one probably generated by oxidation followed by hydroxylation whereas in rats, monohydroxylated metabolites predominated. For example, the second most frequent metabolite in rats was barely detectable in humans (21.6 compared to 0.6%). The relative proportions are given below:

Table 1. Comparison of quantities of human and rat metabolites of cycloastragenol

		Contribution to total metabolites (%)	
Compound/metabolite	**Retention time (min)**	**Rat**	**Human**
Cycloastragenol	15.8	-	-
M1	4.2	7.0	7.1
M2	6.1	8.0	7.6
M3	7.7	13.8	42.5
M4	8.5	13.9	15.6
M5	8.9	21.6	0.6
M6	10.2	26.9	18.2
M7	10.6	9.0	8.3

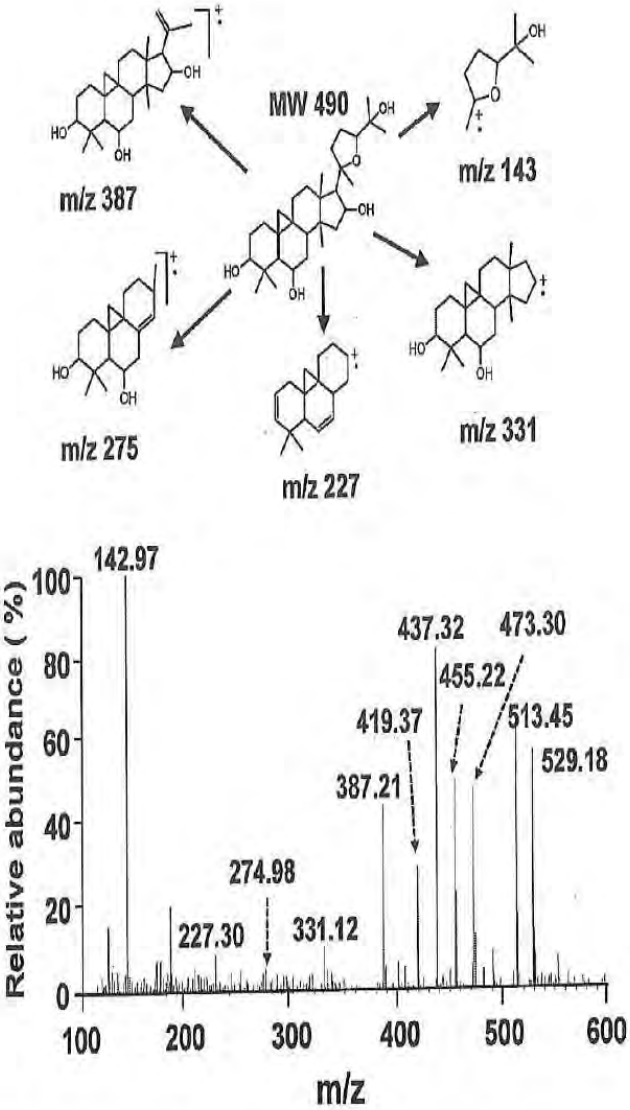


Fig. 5. The CID mass spectrum and proposed fragmentation pathways for CAG
The molecular ions of CAG ($[M+K]^+$ *m/z* 529, $[M+Na]^+$ *m/z* 513) were fragmented by 50% collision energy to generate representative product ions. The major fragmentation pathways for CAG include the cleavage on C17 position which produced the base peak ion *m/z* 143 and ion *m/z* 331, and the loss of water which formed ions $[M-H_2O+H]^+$ *m/z* 473, $[M-2H_2O+H]^+$ *m/z* 455, $[M-3H_2O+H]^+$ *m/z* 437 and $[M-4H_2O+H]^+$ *m/z* 419. The opening of furan ring resulted in ion *m/z* 387, while that of the D carbon ring resulted in ions *m/z* 275 and 227.

Fig 2. Proposed fragmentation pathway for cycloastragenol following hepatic metabolism – taken from Zhu *et al*., 2010

14. From these studies, it was suggested that efficient first pass-metabolism and extensive hepatic metabolism meant that oral bioavailability of cycloastragenol would be low. In support it was noted that, following ingestion of 5-100 mg cycloastragenol TA-65 by human volunteers (0.08-1.67 mg/kg bw for a 60 kg individual), the plasma levels of cycloastragenol were low, being in the range 1-20 nM (Harley *et al*., 2011).

This information is described as unpublished data and no further details are provided, for example, on the levels of cycloastragenol metabolites.

Toxicology

15. The toxicological data supplied in the submission consisted of a 13 week (with a 4 week recovery period) feeding study of cycloastragenol TA-65 in rats, a 13 week ip/iv study in rats and dogs of a Radix Astragali product which contained astragalus polysaccharides and astragalus membranaceous saponins which have cycloastragenol as the aglycone. No treatment related adverse effects were reported, although it should be noted that histopathological examination of the tissues was not conducted.

Genotoxicity data

16. A number of genotoxicity tests were conducted on cycloastragenol TA-65.

Bacterial Reverse Mutation Assay

17. Using a plate incorporation assay, the test article was examined in 2 phases. Firstly, an initial toxicity/mutation assay was conducted to establish the dose range and a confirmatory mutagenicity study. In the initial study, the test article was dissolved in DMSO and tested at 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate with and without S9 metabolic activitation, precipitate was observed at ≥1500 µg/plate and no appreciable toxicity was observed. The confirmatory assay was conducted using doses of 50, 150, 500, 1500 and 5000 µg/plate; precipitate was again observed beginning at 1500 µg/plate. The *salmonella* strains tested were TA98, TA100, TA1535, and TA1537 and Escherichia coli tester strain WP2 *uvr* A in the presence or absence of Aroclor-induced rat liver S9. The study was conducted to GLP standards.

18. The data sets for TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response were ≥ 3 times the mean vehicle control, while for strains TA98, TA100 and WP2 *uvr* A; these were judged positive if the increase in mean revertants at the peak of the dose response were ≥ 2 times the mean vehicle control.

19. The results for the preliminary experiment are tabulated below:

-65 in the absence of metabolic activation (S9)

	Revertants per plate ± SD (2 plates per dose level)				
Concentration µg/plate	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	13 ± 2	148 ± 17	14 ± 6	13 ± 1	14 ± 2
1.5	21 ± 2	160 ± 12	12 ± 1	12 ± 0	17 ± 2
5.0	20 ± 0	164 ± 26	13 ± 1	12 ± 1	17 ± 5
15	16 ± 4	167 ± 1	12 ± 6	14 ± 1	17 ± 1
50	21 ± 6	147 ± 5	14 ± 3	16 ± 2	14 ± 4
150	16 ± 1	174 ± 2	14 ± 1	18 ± 2	16 ± 0
500	19 ± 4	175 ± 27	9 ± 1	15 ± 1	14 ± 2
1500	15 ± 3 NP	153 ± 35 NP	13 ± 2 NP	13 ± 1 NP	7 ± 1NP
5000	9 ± 1 IP	127 ± 9 IP	9 ± 1 IP	9 ± 0 IP	8 ± 0 IP
Positive control	2NF	SA	SA	9AAC	MMS
	181 ± 27	533 ± 15	367 ± 7	43 ± 2	367 ± 7

NP -Non-interfering precipitate, IP -Interfering precipitate, 2-NF- 2-nitrofluorene, SA- sodium azide, 9-AAC- 9- aminoacridine, MMS- methyl methane sulfonate

Table 3. Preliminary assay for cycloastragenol TA-65 in the presence of metabolic activation (S9)

	Revertants per plate ± SD (2 plates per dose level)				
Concentration µg/plate	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	34 ± 7	174 ± 1	17 ± 1	11 ± 1	12 ± 0
1.5	32 ± 8	173 ± 4	11 ± 6	9 ± 4	14 ± 0
5.0	23 ± 6	155 ± 42	12 ± 1	12 ± 4	14 ± 5
15	26 ± 6	170 ± 4	18 ± 6	13 ± 1	15 ± 4
50	27 ± 8	163 ± 23	15 ± 1	8 ± 1	14 ± 1
150	32 ± 10	174 ± 0	13 ± 6	11 ± 0	18 ± 2
500	36 ± 9	171± 5	11 ± 10	14 ± 0	10 ± 3
1500	23 ± 1 NP	158 ± 21 NP	10 ± 3 NP	13 ± 2 NP	6 ± 3NP
5000	19 ± 4 IP	150 ± 1 IP	13 ± 0 IP	9 ± 1 IP	8 ± 0 IP
Positive control	2AAT	2AAT	2AAT	2-AAT	2-AAT
	365 ± 42	965 ± 11	121± 8	221 ± 2	228 ± 82

NP -Non-interfering precipitate, IP -Interfering precipitate, 2- aminoanthracene.

20. The results for the main experiment are tabulated below:

Table 4. Main experiment – S9 for cycloastragenol TA-65 in the absence of metabolic activation (S9)

	Revertants per plate ± SD (3 plates per dose level)				
Concentration µg/plate	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	15 ± 2	140 ± 11	11 ± 1	5 ± 2	18 ± 1
50	14 ± 3	137 ± 9	12 ± 4	6 ± 2	16 ± 7
150	15 ± 2	140 ± 9	15 ± 2	7 ± 1	10 ± 2
500	15 ± 2	136 ± 4	11 ± 3	9 ± 2	12 ± 3
1500	16 ± 2 NP	141 ± 20 NP	13 ± 1 NP	6 ± 2 NP	12 ± 2NP
5000	9 ± 1 IP	95 ± 9 IP	7 ± 2 IP	7 ± 1 IP	10 ± 1 IP
Positive control	2NF	SA	SA	9AAC	MMS
	151 ± 5	549 ± 44	312 ± 74	1545 ± 318	148 ± 4

NP -Non-interfering precipitate, IP -Interfering precipitate, 2-NF- 2-nitrofluorene, SA- sodium azide, 9-AAC- 9- aminoacridine, MMS- methyl methane sulfonate

Table 5. Main experiment for cycloastragenol TA-65 in the presence of metabolic activation (S9)

	Revertants per plate ± SD (3 plates per dose level)				
Concentration µg/plate	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	26 ± 6	166 ± 10	12 ± 2	7 ± 2	14 ± 1
50	23 ± 3	155 ± 17	14 ± 5	7 ± 2	14 ± 2
150	22 ± 5	183 ± 9	14 ± 2	7 ± 2	14 ± 4
500	21 ± 2	188 ± 11	10 ± 2	8 ± 2	17 ± 5
1500	19 ± 6 NP	176 ± 13 NP	11 ± 3 NP	5 ± 3 NP	13 ± 2NP
5000	10 ± 2 IP	114 ± 9 IP	6 ± IP	5 ± 1 IP	10 ± 2 IP
Positive control	2-AAT	2-AAT	2-AAT	2-AAT	2-AAT
	489± 139	910 ± 89	186 ± 32	96 ± 32	703 ± 113

NP -Non-interfering precipitate, IP -Interfering precipitate, 2- aminoanthracene.

21. No positive results were found for cyclastragenol TA-65 in the presence or absence of metabolic activation. The results were within the range of the historical control and the positive control compounds produced clear positive results, also within the range of the historical control. It was concluded that cycloastragenol TA-65 was negative in the bacterial reverse mutation assay.

In vitro chromosomal aberration assay in CH V79 cells

22. An *in vitro* chromosome aberration assay was conducted in Chinese Hamster V79 cells to assess the ability of cycloastragenol TA-65 to induce structural chromosome aberrations. Two experiments were conducted using slightly different concentration ranges and treatment times. In experiment 1, the concentrations used were 0.05, 0.1 and 0.125 mM and 0.05, 1.25, 1.50, 1.75 and 2.00 mM in the absence and presence of metabolic activation respectively. In experiment 2, the

concentrations used were 0.065, 0.07 and 0.075 for 20 hours in the absence of activation and 0.4, 1.0, 1.6, and 2.1 mM for 4 hours in the presence of activation before preparation of the chromosomes. Several criteria for a positive result were noted. These were a clear and dose-related increase in the number of cells with aberrations and/or a biologically relevant response for at least one of the dose groups which was higher than the laboratory negative control range (0-4% aberrant cells, with and without activation. The assay was conducted to GLP standards and in accordance with OECD guideline 473.

23. In experiment 1, precipitation was observed at ≥0.1mM and ≥ 1.25 mM in the absence and presence of activation respectively. In experiment 2, precipitation was observed at all doses tested but only in the presence of activation. In experiment 1, toxic effects of the test article were seen at ≥0.1mM and ≥ 1.50 mM in the absence and presence of activation respectively. In experiment 2, toxic effects were seen at ≥0.075 mM and ≥ 1.0 mM in the absence and presence of activation respectively.

24. In both experiments, the aberration rates were within the range of the historical controls (0-4%) in the absence of metabolic activation.

25. In the presence of activation, inconsistent results were found in both experiments but the mean values for chromosome aberrations increased above the historical data of the negative control only at 1.50 mM in experiment 1. This is stated to be 0-4% but concentrations other than 1.5 mM do exceed this, albeit not by much. The relevant tables are attached at Annex 3 with some details summarised below. The positive control substances ethylmethane sulfonate and cyclophosphamide induced distinct and biologically relevant increases in the number of chromosome aberrations.

Table 6. Summary of results for cycloastragenol TA-65 in the presence of metabolic activation – mean % aberrant cells

Experiment 1	Treatment time (h)	Mean % aberrant cells (inc gaps)	Mean % aberrant cells (exc gaps)
Control	4	3.0	1.5
Solvent control	4	3.0	1.5
Concentration mM			
0.05	4	4.5	2.5
1.25	4	3.5	1.5
1.50	**4**	**8.0**	**5.3**
1.75	4	5.0	2.0
2.00	4	3.5	1.0
CPA	4	10.5	8.0
Experiment 2			
Control	20	4.0	2.0
Solvent control	20	3.5	2.5
Concentration mM			
0.4	20	3.0	1.0
1.0	20	5.0	2.0
1.6	20	3.0	1.8
2.1	20	4.3	2.3
CPA	20	14.0	11.5

26. Examination of the data on individual aberrations in experiment 2 (in the presence of metabolic activation) suggests a small, increasing trend in the number of chromosome aberrations as tabulated below:

Table 7. Structural chromosomal aberrations. Experiment 2 - cycloastragenol TA-65 with metabolic activation.

	Scored cells	Polyploid cells	Aberrant cells Inc.gaps	Aberrant cells Exc.gaps
Control	100 100 **Total-200**	1 1 **2**	4 4 **8**	2 2 **4**
Solvent control	100 100 **Total-200**	0 2 **2**	5 2 **7**	4 1 **5**
0.4	100 100 **Total-200**	0 0 **0**	2 4 **6**	1 1 **2**
1.0	100 100 **Total-200**	0 0 **0**	3 7 **10**	3 1 **4**
1.6	100 100 **Total-200**	0 0 0	8 4 **12**	7 0 **7**
2.1	100 100 **Total-200**	1 3 **4**	6 11 **17**	2 7 **9**
CPA positive control	100 100 **Total-200**	0 1 **1**	11 17 **28**	8 15 **23**

The complete tables are included in Annex 3

27. In both experiments, the most frequent type of aberrations was breaks.

28. No biologically relevant increases in the number of polyploid cells compared to the controls were found in cells treated with cycloastragenol-TA65

Table 8 - Historical data- negative control

	Number of Aberrant cells			
	Metabolic activation			
	with		*Without*	
	+ gaps	- gaps	+ gaps	- gaps
Mean [%]	4.3	2.0	3.6	1.5
SD	1.43	0.81	1.55	0.82
Relative SD	33.5	41.4	42.4	56.6
Min [%]	1.0	0.0	0.5	0.0
Max [%]	8.5	4.0	9.0	4.0
No of assays	181	181	181	181

Table 9 - Historical data- positive control

	Number of Aberrant cells			
	Metabolic activation			
	with		*Without*	
	+ gaps	- gaps	+ gaps	- gaps
Mean [%]	13.1	9.7	13.1	9.7
SD	2.29	1.66	2.70	1.96
Relative SD	17.4	17.1	20.7	20.3
Min [%]	8.5	8.0	8.5	8.0
Max [%]	23.0	16.0	26.5	20.5
No of assays	172	172	172	172

29. It was concluded that since the clastogenic effect was “relatively moderate” and was in the concentration range where precipitation occurred and, as there was no dose-response relationship, the final result of the assay was considered to be equivocal.

In vivo erythrocyte micronucleus assay.

30. For this study an initial range finding study was conducted to determine the maximum tolerated dose which was deemed to be 2000 mg/kg TA-65 (dissolved in cottonwood oil and administered i.p.). For the main experiment, the doses used were 2000, 1000 and 400 mg/kg bw (the MTD and 0.5 and 0.2 MTD) administered i.p. The dose was given i.p. although the product is taken orally; this was presumably done to ensure exposure of the bone marrow although this is not stated. Toxicity was apparent at the top dose level and to a lesser degree at the lower doses. Five animals of each sex were used per dose group, along with a vehicle control and a positive control of cyclophosphamide. The study was conducted to GLP standards and in accordance with OECD guideline 474.

31. A total of 10000 polychromatic erythrocytes were scored for micro-nucleated immature erythrocytes. The negative controls were within the range of historical control data and the results for the test article were with the corresponding negative control range. No biologically relevant increase in micronuclei was found in any of the treatment groups. However, cyclophosphamide treatment resulted in a significant increase in micronuclei frequency.

32. Exposure times were 44 hours, but with additional exposure of 72 hours for the top dose group and the negative control. The findings are tabulated below:

Table 10. Percentage micronuclei after 44h exposure

	Dose mg/kg	MN (% ± SD)	Mean Relative PCE
Males	0	0.18 ± 0.07	3.18
Females	0	0.22 ± 0.09	3.77
Males	400	0.20 ± 0.10	3.06
Females	400	0.17 ± 0.07	2.10
Males	1000	0.19 ± 0.10	2.68
Females	1000	0.12 ± 0.03	2.54
Males	2000	0.19 ± 0.10	2.56
Females	2000	0.14 ± 0.07	2.92
Males	CPA	1.75 ± 0.74	2.30
Females	CPA	1.34 ± 0.56	2.12

Table 11. Percentage micronuclei after 68h exposure

	Dose mg/kg	MN (% ± SD)	Mean Relative PCE
Males	0	0.24 ± 0.06	3.28
Females	0	0.20 ± 0.06	3.89
Males	2000	0.16 ± 0.03	0.96
Females	2000	0.13 ± 0.05	1.03

Carcinogenicity

33. An unpublished non GLP study monitored the effect of TA-65 on tumour growth in mice in which 4 different human cancer cell types had been xenografted. In this study, groups of 10 female nude mice aged 6-8 weeks were inoculated sub-cutaneously with a standard number of cells. They were then treated with TA-65 dosed by gavage at 5 mg/kg bw/day or vehicle control for 40 days starting on the day of cell inoculation. The tumour cells were lung (H460), colon (H29) breast (MDA-MB-435) and prostate (PC3). The treatment had no significant effect on body weight or tumour growth (incidence or size) in any of the groups. In addition to the growth curves of the tumours, an additional “rough” analysis was presented in which the mean growth of the treatment was divided by the mean growth of the control. Presented as a percentage, this suggested that in the MDA-MB-435 and HT-29 inoculated groups, tumour growth was slightly faster in the TA65 treated group compared to the controls, whereas for PC3 and H460 tumour growth was slightly faster in the control group. It was noted that the analysis was intended to determine whether one of the treatments had a slight but non-statistically significant effect.

34. The applicant also referred to the published study by Bernardes de Jesus *et al* (2011). In this study, which was designed to investigate the effects of TA-65 on telomeres rather than being a regulatory study, mature (1 year old) and aged (2 year old) female C57BL/6JOlaHsd mice were given fruit mash containing 25 mg/kg TA-65 or vehicle control for four months. Three months after treatment had stopped, average telomere length in the peripheral blood leucocytes of the treated animals

was unaffected but there was a significant decrease in very short telomeres (< 2, 3 and 4 kb) in both age groups indicating that TA-65 had a significant capacity to promote rescue of short telomeres.

35. In the same study (Bernardes de Jesus *et al*., 2011), TA-65 was also reported to improve biomarkers of aging, including glucose tolerance, liver steatosis, osteoporosis and skin fitness (increased sub-cutaneous fat content and epidermal layer in the 1 year old mice). A pathological analysis was conducted in all the animals at the time of death (n=14 controls and n = 22 treated). The incidence of malignant tumours was similar at the time of death, with the treated group having a slightly decreased incidence of sarcomas and slightly increased incidence of lymphomas (18 and 64% compared to 36 and 50% respectively). An increased incidence of liver tumours (lymphomas and histiocytic sarcomas) was observed in the treated group (60%) compared to the controls (42%) but this was not statistically significant.

ACNFP response and applicants reply

36. The ACNFP expressed concern that the tumour incidence following consumption of the ingredient was high and stated that "the metabolism of the novel food ingredient should not be considered separately from carcinogenicity as the metabolites are carcinogenic"[2]. It was also pointed out that the applicant had stated that the metabolism of TA-65 (as observed in the microsome studies) was qualitatively similar in humans and rats but the evidence suggested it was quantitatively different. The ACNFP response is attached at Annex 4.

37. The applicant responded to the comments made by the ACNFP making the following points (see below). The company's response is also attached at Annex 4.

Metabolism – It was reiterated that the *in vitro* studies (Zhu *et al*., 2010) suggested that oral bioavailability was low due to extensive first pass metabolism. This was supported by unpublished work all showing low levels of cycloastragenol in the serum of volunteers (Harley *et al*., 2010). These data showed cycloastragenol levels peaked 3 hours after dosing and were back to baseline by12-13 hours. However, no comments were made on the fate of the metabolites.

Telomerase mechanism and carcinogenesis- It was pointed out that the findings of the genotoxicity studies were negative and that interpretation of the Bernardes de Jesus *et al*., 2011 study was limited, due to the small numbers used and the pooled results from mice of different ages. It was also noted that the dose used in this investigation was 200 times the recommended human dose. Overall, since the genotoxicity and carcinogenicity data on TA-65 were essentially negative, a lifetime feeding study was not justified.

38. Annex 4 also includes a document which combines the original comments made by ACNFP as well as the views of an ACNFP toxicologist on the applicant's response.

[2] Not all the ACNFP documentation is available, so it is unclear whether this statement is based on the findings of Bernardes de Jesus *et al*., 2011 or other information/discussions.

Additional information

Additional data from studies on TA-65

In vitro

39. It was reported that TA65 was capable of increasing average telomere length and decreasing the percentage of critically short telomeres and of DNA damage in haplo-insufficient mouse embryonic fibroblasts that harbour critically short telomeres and a single copy of the telomerase RNA *Terc* gene (G3 *Terc* $^{+/-}$ MEFs) (Bernardes de Jesus *et al*., 2011). TA-65 did not cause telomere elongation or rescue DNA damage in similarly treated telomerase deficient G3 *Terc* $^{-/-}$ littermate MEFs. The results indicated that TA-65 treatment resulted in telomerase-dependent elongation of short telomeres and rescue of associated DNA damage, thus demonstrating that TA65 mechanism of action is through the telomerase pathway.

40. Low nanomolar levels of TA-65 moderately activated telomerase in human keratinocytes, fibroblasts and immune cells in culture, at similar plasma levels as occurred in pilot pharmacokinetic studies (Harley *et al*., 2011).

41. Exposure of $CD8^+$ lymphocytes taken from HIV-infected donors to TAT retards telomere shortening, modestly increases proliferative potential and enhances cytokine/chemokine production and antiviral activity (Fauce *et al*., 2008). The enhanced antiviral effects were abrogated in the presence of GRN163L a potent and specific telomerase inhibitor, suggesting that TAT2 acts primarily through telomerase activation.

In vivo

42. Supplementation with 25 mg/kg TA65 in fruit mash for four months increased the levels of mouse telomerase reverse transcriptase by 10 fold in the liver when measured 3 months post-treatment, with modest increases being apparent in tissues such as kidney, lung or brain although these were not statistically significant (Bernardes de Jesus *et al*., 2011). No significant differences were apparent in the haematological parameters in the treated mice of either age, there were significant increases in the number of red blood cells and platelets and in the levels of haemoglobin and the haematocrit of the treated 2 year old mice compared to their age matched controls.

43. In zebra finches treated with TA-65 in water for 2 months, red blood cell telomere length and flight feather regeneration were increased compared to the controls (Reichert *et al*., 2014). It was concluded that TA-65 could rescue telomere length with a “global positive effect on the proliferating capacity of cells, in this case epidermal cells. The authors stated that long telomeres could reflect high telomerase activity, and in doing so be a good predictor of greater telomerase dependant tissue regeneration which could explain variation in organism quality and longevity.

Human

44. A group of human volunteers undertook the PattonProtocol-1 health program which included a dose of 10-50 mg TA-65/day, a comprehensive dietary supplement pack and physician counselling and laboratory tests at baseline and every 3-6 months thereafter (Harley *et al*., 2011). The number of evaluable subjects was 114, 43, 59, 27 and 37 at baseline, 3, 6, 9 and 12 months respectively. The subjects had a mean age of 63 ± 12 years and were 72% male. Compared to baseline, a decrease in the numbers of senescent cytotoxic ($CD8^+/CD28^-$) T cells (1.5, 4.4, 8.6 and 7.5% at 3, 6, 9 and 12 months respectively) was measured, though this was not significant. The numbers of natural killer cells at 6 and 12 months were also decreased compared to baseline. Most of the decreases were seen in cytomegalovirus (CMV) seropositive subjects. In a subset of 13 subjects, the mean telomeric length in leukocytes was not changed at 12 months compared to baseline, but there was a significant reduction in the percentage of short telomeres (<4kbp). It was concluded that the program lengthened critically short telomeres and remodelled the relative proportions of circulating leukocytes of CMV^+ subjects towards the more "youthful" profile of CMV^- subjects.

Telomeres and telomerase

45. Telomeres are complexes of DNA and protein which cap the end of chromosomes, protecting them from degradation. Telomeres consist of many kilobases of TTAGGG nucleotide repeats.

46. The ends of telomeres form a t-loop which may be formed by strand invasion of the 3' single strand overhanging into the preceding double stranded telomeric DNA (Buseman *et al*., 2012). The loop is then stabilised by a six-protein complex called shelterin, which functions to maintain telomere length, promote t-loop formation, recruit telomerase to telomeric ends and to protect the ends of chromosomes from being recognised as DNA damage.

47. When a few telomeres become critically shortened there is a growth arrest state at which time DNA damage signalling and cellular senescence is triggered. It is possible that telomeres reach a critically short length that is no longer competent to assemble into the t-loop structure and, that even a few uncapped telomeres in the cell is enough to initiate replicative senescence.

48. Telomerase is an enzyme which counteracts telomere shortening by synthesizing new telomeric repeats at the end of the chromosomes. In cells without telomerase, telomeres progressively shorten in length with each round of cell division due to incomplete replication of the chromosome ends and insufficient activity of the telomere-producing enzyme telomerase. This attrition leads to telomere dysfunction and genomic instability which triggers a DNA damage response that promotes cell cycle arrest and replicative senescence. Functional telomeres must evade both the ATM and the ATR checkpoint pathways, both of which respond to DNA double strand breaks at telomeres.

49. The human telomerase holoenzyme core components are a catalytic reverse transcriptase called human telomerase reverse transcriptase (hTERT) and an

associated template RNA called hTR (human Telomerase RNA). hTR is expressed in all human cells and telomere activity is limited by the expression of hTERT which is only found in cells with detectable telomerase activity. Telomerase extends telomeres by binding to the 3' single stranded end of the telomere end catalysing the addition of a single telomere repeat and translocating to the new terminus. This continues until the holoenzyme dissociates from the telomere. Confirmed and putative telomerase-associating proteins have been described.

50. Evidence suggests that the progressive loss of telomeric repeats of chromosomes may function as an important timing mechanism during the aging process in various species (reviewed Boccardi and Paolissi, 2014). Numerous epidemiological studies show that shorter telomeres in humans are associated with many age related diseases such as cancer, cardiovascular diseases (atherosclerosis, hypertension, myocardial infarction) and cognitive decline, diabetes and overall mortality. Patients with dyskeratosis congenital and idiopathic pulmonary fibrosis display chromosomal instability and accelerated cellular senescence particularly in tissues with high proliferative activity. These hereditary diseases are caused by mutation in telomerase. Whilst telomerase is generally only active in stem and germinal cells, somatic cells without a functional telomerase also show accelerated shortening and dysfunction (reviewed Boccardi and Paolissi, 2014).

51. Rare cells may escape replicative senescence by gaining telomerase expression, which enables them to maintain telomere length and become immortalised with further clonal evolution leading to the acquisition of genetic changes that result in malignancy (Novak, 2009).

Telomeres and cancer

52. The rare cell that can maintain telomeres is then able to grow continuously and this is believed to be a critical step in cancer progression. Such cells tend to have telomere stability (at very short lengths) and telomerase reactivation (Shay and Wright, 2011) suggesting that the initiating cancer stem cell was likely to have a very short telomere when telomerase was reactivated and the cancer cells with stem like markers are likely to have shorter telomeres compared to the bulk of the cells in the tumour.

53. When telomerase is upregulated or reactivated, a number of outcomes are possible. Cells might not express enough telomerase and would not be able to divide long term to become robust malignant cells or telomerase would be expressed in excess and the telomeres would be expected to grow rapidly leading to long telomeres; this is seen in less than10% of primary cancers, and thus it may be of no selective advantage for cancer cells to have more telomerase than needed to maintain telomeres longer than that which protects against DNA damage signalling/end-fusion. What is observed is that the vast majority of human cancer cells have telomeres generally the same or shorter than adjacent normal tissue, suggesting that telomerase is not in excess (Shay and Wright, 2011).

54. It is also generally believed that greatly shortened telomeres in initiated, but still neoplastic, cells (while initially a potent anti-cancer protection mechanism) may eventually promote genomic instability and lead to the development of advanced

disease. It is widely accepted that genetic instability drives malignant transformation and that most human cancer cells have grossly altered chromosome rearrangements. With only a few cellular alterations, the DNA damage signals from telomere shortening (telomere uncapping) would be predicted to be a very potent tumour suppressor pathway since the “damage” could not be repaired in the absence of telomerase. Thus, replicative senescence is likely to initially stop cells from proliferating and progressing to cancer (Shay and Wright, 2011).

55. In the absence of critical checkpoint pathways, genomic instability occurs when telomeres are short, leading to end-to-end fusions, anaphase bridges, the development of aneuploidy and eventually to telomerase reactivation. One possibility is that the re-expression or up regulation of telomerase in cancer reduces the ongoing chromosomal instability that occurs in cells in crisis to a level compatible with viability. However, most benign or emerging cancer cells have several mutations including the reactivation of telomerase and there remains sufficient genomic instability to generate mutational evolution of the malignancy. In summary, telomere shortening may be a common underlying cause of chromosomal rearrangements in cancer (Shay and Wright, 2011).

56. While telomerase does not drive the oncogenic process, it is permissive and required to sustain the growth of most advanced cancers (Shay and Wight, 2011).

57. Normal tissue stem cells show progressive telomere shortening with increased age and telomerase is carefully regulated so that it is not continuously expressed. Thus normal tissue stem cells are telomerase competent but mostly silent while cancer stem cells almost universally constitutively express telomerase. T and B lymphocytes transiently upregulate telomerase (Fauce *et al*., 2008). Such telomerase activity is believed to mitigate the losses of replicative capacity and function arising from chronic antigen stimulation, oxidative stress and cellular aging.

58. Introduction of the catalytic protein hTERT, activated telomerase activity in normal telomerase silent cells and was sufficient to bypass senescence leading to cell immortalisation. It was further shown that ectopic expression of telomerase hTERT in pre-senescent human cells or in cells between senescence and crisis could be immortalised with only the ectopic introduction of hTERT demonstrating that telomeres are mechanistically important in both senescence and crisis. Finally it was demonstrated that hTERT immortalised normal human cells were karyotypically normal and not transformed (Shay and Wright, 2011).

59. However, there are other mechanisms by which telomeres are maintained since studies in model organisms, in which telomerase has been deleted, indicate that multiple recombination-based mechanisms can contribute to telomerase maintenance (Morrish *et al*, 2015). Such mechanisms are likely to be recombination based and use repetitive sequences such as retrotransposons. Tumours lacking telomerase can still maintain telomerase sequences and typically include sarcomas and some types of brain tumours.

60. Telomere length in inbred rodent strains is greater than that in humans and telomere expression is more robust in rodent cells (Shay and Wright, 2011). Telomere biology and its regulation may differ in long and short lived species.

Summary

61. Telomeres are kilobases of nucleotide repeats which occur at the end of chromosomes protecting them from degradation. Telomeres progressively shorten in length at each round of cell division due to incomplete replication and insufficient activity of the telomere-repairing enzyme telomerase. This eventually leads to telomere dysfunction and genomic instability, triggering cell cycle arrest and replicative senescence. Telomere attrition is thought to be important in cellular aging and shortened telomeres have been associated with degenerative diseases in humans. Telomerase is active in human stem and germinal cells, but not in somatic tissue. Some cells may gain telomerase activity and become immortalised, with further clonal evolution leading to the acquisition of genetic changes that lead to malignancy. Cycloastragenol-TA65 has been reported to increase the number of critically short telomeres, but not to increase mean telomere length.

62. *In vitro* investigations suggest that TA-65 is absorbed and undergoes significant first pass metabolism into a number of oxidised and hydroxylated compounds, meaning that the bioavailability of the parent compound is low. There are few data available on the structure of the metabolites. Information from MS analysis suggests that oxidised metabolites and glucuronide conjugates may occur via intestinal metabolism. Data from microsomal studies suggested that up to seven metabolites were produced which were largely monohydroxylated with additional hydroxylation occurring post oxidation. Some cleavage of the ring structure was also proposed. Information of the fate of the metabolites is not available.

63. The ACNFP initially asked the COC to comment on some aspects of an application to place on the market the novel food ingredient cyclastragenol TA-65 a small molecule derived from the plant *Astragalus trojanus*. The COC expressed a number of concerns about the compound and suggested that COM consider the genotoxicity data, both whether it was appropriate given cycloastragenol's proposed mechanism and whether they were content with the view of the manufacturers that cycloastragenol was negative or equivocal for genotoxicity

Questions for COM

64. The COM are asked to comment on the genotoxicity studies provided.

a) Has an appropriate range of studies been conducted, given the proposed mechanism of action of cyclastragenol? If so,
b) Are the results sufficient to conclude that cycloastragenol does not have any genotoxic potential?
c) Any other comments they may have.

Secretariat
May 2015

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Annex 1 to MUT/2015/08

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REQUEST FROM ACNFP FOR ADVICE ON THE NOVEL FOOD INGREDIENT CYCLOASTROGENOL

Application for placing on the market cycloastragenol-TA65 as a novel food.

Publicly available version from:

http://acnfp.food.gov.uk/sites/default/files/mnt/drupal_data/sources/files/multimedia/pdfs/cycloastapp.pdf

Secretariat
May 2015

COMMITTEE ON CARCINOGENICITY OF MUTAGENICITY IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REQUEST FROM ACNFP FOR ADVICE ON THE NOVEL FOOD INGREDIENT CYCLOASTROGENOL

Zhu, J., Lee S., Ho, M.K/C., Hu, Y., Pang H., Ip, F.C.F., Chin, A.C.C., Harley, C.B., Ip, N.Y., Wong, Y.H (2010). *In vitro* Intestinal Absorption and First-Pass Intestinal Metabolism of Cycloastragenol, a Potent Small Molecule Telomerase Activator. Drug Metabolism and Pharmacokinetics, 25, 477-486.

This paper can be downloaded from:

http://repository.ust.hk/ir/bitstream/1783.1-8095/1/25_477.pdf

Secretariat
April 2015

Annex 3 to MUT/2015/08

COMMITTEE ON CARCINOGENICITY OF MUTAGENICITY IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REQUEST FROM ACNFP FOR ADVICE ON THE NOVEL FOOD INGREDIENT CYCLOASTROGENOL

Results Tables from *in vitro* study of chromosome aberration in CH V79 cells

Secretariat
May 2015

Table 4: Experiment I - Structural Chromosomal Aberrations, *with* metabolic activation: 4 h treatment, 20 h fixation period.

Dose Group	Concen-tration [mM]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Types of Aberrations Found: Chromatid types b	f	d	ex	Chromosome types ib	if	id	cx	Other ma	cd
C	0	1	100	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	3	2	1	0	2	0	0	0	0	0	0	0	0	0
		total	200	0	6	3	3	0	3	0	0	0	0	0	0	0	0	0
S	0	1	100	0	3	2	2	0	2	0	1	0	0	0	0	0	0	0
		2	100	0	3	1	2	0	0	0	1	0	0	0	0	0	0	0
		total	200	0	6	3	4	0	2	0	2	0	0	0	0	0	0	0
1	0.05	1	100	1	3	3	0	0	1	1	0	0	0	0	0	1	0	0
		2	100	1	6	2	4	0	2	0	0	0	0	0	0	0	0	0
		total	200	2	9	5	4	0	3	1	0	0	0	0	0	1	0	0
5	1.25	1	100	2	5	2	3	0	0	0	0	0	1	2	0	0	0	0
		2	100	0	2	1	1	0	1	0	0	0	0	0	0	0	0	0
		total	200	2	7	3	4	0	1	0	0	0	1	2	0	0	0	0
6	1.50	1	200	0	13	10	3	0	8	1	0	0	0	0	0	0	1	0
		2	200	0	19	11	10	0	9	0	0	1	0	1	0	0	0	0
		total	400	0	32	21	13	0	17	1	0	1	0	1	0	0	1	0
7	1.75	1	100	0	6	2	4	0	1	1	0	0	0	0	0	0	0	0
		2	100	1	4	2	2	1	1	0	0	0	0	0	0	1	0	0
		total	200	1	10	4	6	1	2	1	0	0	0	0	0	1	0	0
8	2.00	1	100	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
		2	100	0	6	2	4	0	2	0	0	0	0	0	0	0	0	0
		total	200	1	7	2	5	0	2	0	0	0	0	0	0	0	0	0
CPA	0.83 µg/mL	1	100	0	10	9	1	0	5	0	0	6	0	0	0	0	0	0
		2	100	0	11	8	4	0	5	0	0	2	0	1	0	1	0	0
		total	200	0	21	17	5	0	10	0	0	8	0	1	0	1	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
CPA: Positive Control (Cyclophosphamide)
(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Table 7: Main Experiment II - Structural Chromosomal Aberrations, *with* metabolic activation: 4 h treatment, 20 h fixation period.

Dose Group	Concen-tration [mM]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells		Gaps		Types of Aberrations Found									
									Chromatid types				Chromosome types				Other	
					incl. Gaps	excl. Gaps	g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
C	0	1	100	1	4	2	2	0	2	0	0	0	0	0	0	0	0	0
		2	100	1	4	2	2	1	1	0	0	1	0	0	0	0	0	0
		total	200	2	8	4	4	1	3	0	0	1	0	0	0	0	0	0
S	0	1	100	0	5	4	1	0	3	0	0	0	0	0	0	0	1	0
		2	100	2	2	1	1	0	0	0	0	0	0	0	0	1	0	0
		total	200	2	7	5	2	0	3	0	0	0	0	0	0	1	1	0
4	0.4	1	100	0	2	1	2	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	4	1	3	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	6	2	5	0	2	0	0	0	0	0	0	0	0	0
5	1.0	1	100	0	3	3	0	0	1	1	0	0	0	0	0	1	0	0
		2	100	0	7	1	5	1	1	0	0	0	0	0	0	0	0	0
		total	200	0	10	4	5	1	2	1	0	0	0	0	0	1	0	0
8	1.6	1	200	0	8	7	2	0	4	2	0	1	0	0	0	1	0	0
		2	200	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0
		total	400	0	12	7	6	0	4	2	0	1	0	0	0	1	0	0
10	2.1	1	200	1	6	2	5	0	2	0	0	0	0	0	0	0	0	0
		2	200	3	11	7	4	1	6	1	0	2	0	0	0	0	1	0
		total	400	4	17	9	9	1	8	1	0	2	0	0	0	0	1	0
CPA	0.83 µg/mL	1	100	0	11	8	3	0	3	0	0	4	0	0	0	1	0	0
		2	100	1	17	15	2	0	7	0	1	6	0	0	0	1	0	0
		total	200	1	28	23	5	0	10	0	1	10	0	0	0	2	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (DMSO)
CPA: Positive Control (Cyclophosphamide)
(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Annex 4 to MUT/2015/08

Commercial in Confidence

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REQUEST FROM ACNFP FOR ADVICE ON THE NOVEL FOOD INGREDIENT CYCLOASTROGENOL

Comments from ACNFP on the cycloastragenol TA-65 application and response from applicant.

Secretariat
May 2015