Pécs, January 27th, 2004

Final Report of Research

About in vivo examination results performed on gene expression changes by a Flavin7 extract provided by Crystal Institute Kft.

The studies were performed on CBA/Ca male and female mice (CBA/Ca mice are H-2k haplo type ones. They are sensitive to chemical carcinogenic treatments, several tumours are formed in them, e. g. hepatoma, lung, kidney, haematological tumours), by three kinds of treatment protocol; we also measured the impact effectuated on oncological/tumour suppressor genes expression. The Flavin extract was provided by Crystal Institute Kft., the dosage was 3 bwkg/1 ml, determined by them. The dosage was applied in form of intraperitoneal injections.

- 1st experimental protocol: through a single treatment by dimethyl-benz[a]anthracene (DMBA) (which is a known and proven carcinogenic substance, and both by our recent tests and the technical literature it is generally recognized that it provokes overexpression of some oncogenes and tumour suppressor genes) (40 mg/bwkg dosage) we provoked oncological/suppressor gene overexpression then, 24 hours after the DMBA treatment an ip. Flavin dosage was applied. Thereafter 24 hours later gene expressions were tested (for further details see below). This protocol is called „ulterior treatment" in the following by us.
- 2nd experimental protocol: DMBA treatment and Flavin were given at the same time, then we recorded the gene expression changes within 24 hours. („simultaneous treatment")
- 3rd protocol: The animals were given a Flavin treatment first, then 24 hours later they received DMBA. Gene expression changes were tested after another 24 hours. („previous treatment")

The animals were dissected after cervical dislocation at the indicated time (10 male ones and 10 female ones from each group), their thymuses, livers, spleens, kidneys, lungs and mesentherial lymphatic glands were removed. We isolated RNA through a phenol-chloroform method, then the RNA was applied on a Hybond N+ membrane, using Hoefer slot-blotter. The membranes were hybridized by chemiluminescently marked, cloned gene tests, then autoradiographically evaluated, using a Quantiscan software. During our trials we tested expression changes of Ha-*ras* and *c-myc* oncogenes and *p53* tumour oppressor genes.

The results are shown in graphs 1-18. Each diagram shows the results of a given protocol, in an organ, to the 3 examined genes, so accordingly diagrams 1-6 are showing results of the preliminary treatments, diagrams 7-12 are showing results of the simultaneous treatments, diagrams 13-18 however are showing results of the ulterior treatments. Other than details of the experimental groups, gene expressions of untreated controls (applied only vehicles, but DMBA or ip. Flavin were not) and gene expressions of animals treated only by DMBA are also shown in the diagrams. Gene expressions of untreated controls were used as a base of comparison in every case, so it was 100%, however as a result of DMBA treatment, overexpressions were formed, as they are visible in the graphs as well. The principle of the trial was that in case of a Flavin treatment to what extent it is able to lower these overexpressions, that is to diminish the effects of carcinogenic, treatments on the level of gene expression changes.

Male and female animals used in trials did not show any significant differences, therefore sub-divisions by sexes are not shown in the graphs, so that they would be more easily surveyed, and we worked with averages/ means of all the animals instead.

The results, summarized in a few words were these: **the most important alteration to emphasize by all accounts is, that overexpressions significantly diminished as a result of simultaneous treatments, that is to say the Flavin extract can prevent the effects of DMBA on the level of gene expression changes.** The effects of course asserted themselves in different degrees by genes and organs. In case of *c-myc* oncogene there were not any significant effects, overmore expression of c-myc oncogene showed significant differences by organs, too. Nevertheless comparing to DMBA treated positive controls, relatively smaller decrease appeared in trials of livers,

lungs and kidneys. Intensified *p53* and Ha-*ras* expressions provoked by DMBA were decreased more remarkably and consequently by Flavin extracts however. It was statistcally significant and expressive to a high degree in case of livers, lungs, spleens, kidneys and especially in that of thymuses, though there were not any changes like these in the lymphatic glands exclusively.

In case of ulterior treatments Flavin extracts did not appear so effective in the same measure, as in most cases they did not cause any significant gene expression changes in a big way, comparing to positive controls. Practically no effects at all have been shown at c-myc genes, neither in case of Ha-ras appeared any significant alterations (although in livers, kidneys and lymphatic glands some lower expressions were measured, compared to DMBA-treated controls. Compared to positive controls, gene expression of the p53 tumour suppressor was lower in this case and nearly at all organs, and there were quite significant changes especially in lymphatic glands, thymuses and kidneys.

In case of previous treatments there were not any differences between positive controls and gene expressions of treated animals, or rather they appeared to be within the statistical limits of error. P53 suppressor gene was an exception, as it showed a lot lower expressions in spleens and lungs as well, as a result of Flavin treatments, rather than without them. It is worth to mention that in case of the Ha-*ras* oncogene a similar, but a lot smaller effect was traceable.

Summing up the results of the above mentioned things, we can say that **in case of simultaneous treatments Flavin extracts significantly diminished the increased gene expressions porovoked by carcinogenous treatments. In case of ulterior treatments this effect was still traceable, though it was a lot weaker and appeared in less organs. Previous treatments did not appear to be effective in this experimental system.**

If we examine these data broken down according to organs, we can get an answer to the question of organ specificity. As in case of *c-myc* oncogene there were not any significant alterations, it is necessary to analyse the Ha-*ras* and *p53* data instead. In case of the liver all of the three protocols showed some changes (even at the seemingly and essentially uneffective previous treatments), and this fact is important because in the first round most carcinogenic substances get metabolysed in the liver, therefore the changes measured there seem to be very good indicators of early alterations. *P53* suppressor gene showed significantly lower expression in spleens as a result of Flavin treatments, rather than without them. In lungs (except for *p53* at prevoius treatments) and in lymphatic glands the same effect has only succeeded in case of simultaneous treatments. The examination of kidneys presented a decrease of overexpressions as a result of simultaneous or ulterior treatments. In case of thymuses both simultaneous and ulterior treatments appeared to be effective, or rather a decrease of *p53* overexpression is traceable at ulterior treatments.

It is extremely remarkable how a Flavin extract can diminish oncogenic/ tumour suppressor gene overexpressions provoked by DMBA, even after a single dosage. It is worth to mention however that – in spite of the fact that overexpression decreases were significantly expressive in some cases – regarding gene expressions of negative control animals mostly higher gene espressions were measured, even despite of Flavin treatments. This means that single treatments and simultaneous trials have got their limits. In order to be able to have a clear sight of gene expression level changes, first it is practicable to examine the kinetics of these changes, which seem to be expressive to perform in models of in vivo gene expression alteration models used by us, and these should be completed by 24 hour data, or those of 48 and 72 hours as well. On the other hand it is worth trying several different dosages, so that we could find out what effects can be performed by a single Flavin treatment.

An obvious parallel appears to be between the interpretation of examination results, comparing therapeutical and preventive impacts. Evidently an ulterior treatment might be the analogy of therapeutical intervention at the present model, however parallelling both simultaneous or previous treatments with prevention in fact, we can say that we have been meeting continuous carcinogenic expositions in our lives, therefore while applying a substance with the purpose of cancer prevention for a longer period of time, after all we are applying a simultaneous and previous dosage, too. Previous and simultaneous dosages cannot be separated from each other really so sharply. It would be useful to examine the effects of a longer term, 1-2 week period previous treatments as well.

If we try to draw conclusions reagarding the existence of therapeutical effects of Flavin extracts, it is impossible to prove it, nor refute it on the basis of the present data, though they relate to the supposition that ulterior treatments seemed to be able to decrease the effects provoked by DMBA overexpressions given 24 hours sooner at certain organs and oncogenes. This appears to be encouraging in the regards of giving perhaps different or repeated dosages, so that we could achieve more efficient expression decrease. It is however a hypothesis and cannot be drawn from the present data, but could be researched through further trials that will be proprosed by us at the end of this report.

Regarding preventive effects, the present data show a lot more, too, whereas simultaneous dosages provided considerable protection against DMBA gene expression level effects. This fact calls the attention to the choice that (in case this is possible to perform in an examination of an animal model with a single dosage) in case of ordinary dosages (or oral application) it may be supposed that it has a tumor preventive effect. This hypothesis is based on the fact that a simultaneous treatment was able to prevent the formation of greater DMBA overexpression level effects. If this effect continues to exist in case of repeated application as well, it is feasable that it could provide protection against permanent carcinogenic impacts for the body. Though what measure it might be, it could be only determined by trials modelling real circumstances.

As a result of our research we can say that in the animal model applied by us (CBA/Ca H-2 mice, *in vivo* gene expression changes) the application of one single Flavin extract has decreased gene expression level effects of chemical carcinogenes. On the basis of the foregoing (as some effects were experimented in the basic protocol system) it seems to be practical to go on paying attention to further examination of Flavin extracts. **Based on theoretical considerations these data may support the existence of a possible therapeutical, or morover a potential tumour preventive effect.** To be able to say it certainly and considering to have a more familiar view of all this however, further trials seem to be practical to perform. In our opinion the chief tendencies might be the following:

- Studies of kinetics of gene expression changes in time, following a single dosage, in a model similar to the above mentioned one, but extended to the gene expressions measured after 48 or 72 hours as well. This will make possible that effects appearing perhaps later could be registered, too.
- Effects of a single dosage in the previous model, completed with an application of a different dosage. This kind of experiment might give an answer what the smallest dosage is to be satisfactory to provoke the necessary impacts, or what connection there is between the increase of the dosage and the provoked reaction. It might be proved also, how far it is sensible (necessary) to increase the dosage in order to be able to achieve an optimal gene level effect. The impact of various dosages can be complemented or combined by some kinetical examinations performed after 48 or 72 hours of time.
- In order to be able to complete the results of previous treatments it seems expediential to research the possible impacts of a longer term (7-12 day) previous treatment.
- In regards of the dosages it is suitable to try an analogous method as in human application, that is giving a Flavin product orally to animals. This should be compared with results received from ip. dosages (these should take first place among examine protocols), and this could provide some information, whether the way of dosage has an impact on organ specificity, the intensity of impact, duration in time, constancy, or whether there appear to be any possible differences in sexes or not.
- Following „short-term” examinations and the knowledge provided by them, it seems practical to research on continuous, long term dosages („long-term” examination), with the aim of measuring gene expression changes and, on the other hand the possible decrease of the number of chemical carcinogene induced tumours might be normative. In case of research for gene expression changes, first impacts of a single, then a greater dose DMBA treatment should be studied, on the other hand effects of dosages of repeatedly given carcinogenic dosages on a long term.

The listed and the advised examinations for wider recognition of the biological impacts of Flavin extracts would be destined to describe possible uses and limits of the substance. Of course it seems sensible to perform human epidemiological – molecular epidemiological research as well, however only by having a thorough knowledge of all the processed data of the animal studies, too.

So far on the basis of the achieved results – and our proposals – you decide to continue your studies, we will be available in the future as well, in connection with the possible studies. In this case, depending on the needs and possibilities, it seems practical to have further personal talks in order to settle research strategies for the future. With the remark that for satisfactory professional preparation we advise that by all means.

Yours truly:

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