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14. ABSTRACT
The major goal of the program is identification of novel radioprotectants of Mycoplasma origin. The program consisted of two branches: screening of Mycoplasma strains for anti-apoptotic factors; and testing of predicted candidates for radioprotective activity. Both approaches were successfully implemented and yielded positive results. First, we have found and are currently characterizing a strong anti-apoptotic activity in the lysates of M. arginini. Second, an extremely potent radioprotectant, named CBLB601, structurally related to Mycoplasma lipopeptides was discovered. We have optimized CBLB601 administration regime for reaching maximal radioprotection as well as embarked upon characterization of its toxicological and pharmacological profiles. CBLB601 showed remarkable efficacy, being capable of protecting 100% of treated mice from supralethal 10Gy total body irradiation dose with evident rescuing lymphoid organs. Initial structure-function analysis of CBLB601 led to the understanding that radioprotection is conferred by stimulation of TLR2/TLR6 but not TLR2/TLR1 dimers.

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Final Progress Report

on DARPA-funded research program

Title: **Tissue protecting antidotes from anti-apoptotic factors of *Mycoplasma***

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Solicited applications addressed by the program:

- Accelerating the healing response of tissue;
- Biological approaches for maintaining the warfighter's performance and capabilities in the face of harsh battlefield conditions;
- Biological approaches for increasing medical survival on the battlefield;
- Biological approaches for enabling faster recuperation from battlefield injury;
- Biological approaches for minimizing the after-effects of battle injuries.

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Statement of the problem studied

Severe pathological consequences of battlefield injuries can be largely determined by acute response of damaged tissues, involving excessive loss of cells through the mechanism of programmed cell death (apoptosis). The risk of death or disability can be greatly reduced by suppressing such a deadly reaction. We proposed to generate tissue-protecting antidotes using natural anti-apoptotic factors of *Mycoplasma*, microbial parasites that frequently cause asymptomatic chronic infections in humans. These factors are adapted by millions of years of evolution to protect mammalian tissues from injuries caused by the persistence of the parasite. The proposed program is aimed at identification and production of such factors and their comprehensive testing as tissue protecting agents against injuries, caused by ionizing radiation, acute chemical poisoning, heat shock and hypoxia.
The major long-term goal of this broad program is to develop new drugs that can reduce immediate or remote fatalities resulting from combat-associated injuries and caused by severe physical (radiation, heat shock, burns), chemical (poisoning) or biological (infectious pathogens) stresses. These antidotes will act by targeting natural mechanisms of cell response to external and internal stresses, namely p53 and NF-κB signaling pathways.

Summary of the most important results

Introduction

The major goal of the current program is to develop a new class of radioprotectants based on bacterial products involved in host-parasite interaction and capable of tissue protection. The existence of such factors follows from numerous virological studies indicating that the vast majority of viruses encode powerful anti-apoptotic factors essential for their successful replication. We hypothesized that the ability to suppress death of host cells is not limited to viruses and is a general property of parasites. We further hypothesized that extracellular parasites are likely to develop, during millions of years of co-evolution with their hosts, secreted cell-protecting factors that can be viewed as prototype tissue-protecting drugs especially effective in tissues that are targeted by the particular parasite. As a proof of principle for this approach, we developed CBLB502, a tissue-protecting drug derived from flagellin, secreted protein of *Salmonella*. Flagellin and CBLB502 are capable of activating pro-survival NF-kB pathway in the cells via interaction with Toll-like receptor 5 and serve as powerful protectants of small intestine from lethal doses of ionizing radiation.

Based on these preliminary studies and other evidence on anti-apoptotic activities of parasites (see the original proposal for references), we initiated a broad program aimed at systemic search for tissue protecting factors of mammalian parasites. Among the array of parasites inhabiting mammalian tissues we focused on those that better fit the following criteria:

1. **Extracellular location**. This is an important criterion that increases the likelihood of isolating factors that act through receptors or have another mechanisms of effective access to their cellular targets being added to the cells, thereby being more suitable for pharmacological applications

2. **Latency**. Most successful parasites are those that do not cause any harm to their environment which presumably involves active mechanisms of tissue protection. Hence, our hypothesis is that the more benign is the parasite, the more effective mechanisms of tissue protection it might employ.
3. **Indications of cell protection**. The existence of such information greatly increases the likelihood of isolation desirable factor. This may include indirect evidence such as the ability to activate pro-survival (NF-kB, Bcl2, etc.) or suppression of death pathways (p53, Fas, etc.).
4. **Desirable tissue specificity**. Mammalian tissues differ dramatically in their sensitivity to ionizing radiation. General radiosensitivity of mammalian organism is determined by radiosensitivity of a few tissues, such as hematopoietic system and GI tract, with lungs and kidneys being the next most sensitive organs. Hence, we will give preference to those parasites that target these tissues.
5. **Availability of experimental model and protocols for cultivation** would facilitate technical aspects of isolation of tissue-protecting factors.
6. **Degree of characterization and complexity of the genome**. The availability of whole genome sequence and information of the physiology and metabolism of the parasite could be a very serious factor facilitating the identification of ultimate tissue protecting agents.

Among the microbial parasites of mammals, *Mycoplasma* is the one that fits the majority of the above criteria. The present program is devoted to the exploration of this putative source of tissue protecting factors. The main objective of the first stage of the program is to establish proof of principle by demonstrating our ability to isolate radioprotective factor of *Mycoplasma* origin. The milestone of the funded first Phase of the proposed study was to identify and evaluate *in vivo* in mice at least one radioprotective factor of *Mycoplasma*.

One of the branches of this program already at its very initial stages (1st Phase conducted in October 2004-May2005) has luckily led us to the discovery of an extremely potent radioprotectant structurally related to *Mycoplasma* lipopeptides. This PAM2-lipopeptide, designated CBLB601, is viewed by us as the basis for development of a new class of radioprotectants of Protectan600 series. CBLB601 showed remarkable efficacy, being capable of protecting 100% of treated mice from supralethal 10Gy total body irradiation dose with evident rescuing lymphoid organs, like spleen and thymus. At the second stage (May-November, 2005), we were mainly focused on optimization of CBLB601 efficacy, its toxicological and pharmacological profiles as well as attempted to decipher which structural features of CBLB601 confer radioprotection and through what mechanism. Initial structure-function analysis of CBLB601 led to the understanding that radioprotection is conferred by stimulation of TLR2/TLR6 but not TLR2/TLR1 dimers.

Since we have reached the Phase I milestone (see description below), the program now enters its next phase that involves four major directions of study: (i) optimization of efficacy, toxicity and pharmacological profile of the identified radioprotectant CBLB601; (ii) identification and generation of additional activators of TLR2/TLR6 heterodimers and assessment of their radioprotective properties; (iii) - determination of cellular and molecular mechanisms of activity and the targets of CBLB601 and other TLR2/TLR6 ligands; (iv) identification and further characterization of anti-apoptotic activity found in the lysates of *M. arginini*; and (v) extension of the search for tissue protecting factors of other *Mycoplasma* species using the developed methodology.

Major results

General strategy and the outline of our experimental plan are shown in Figure 1. Our plan included studies in two parallel directions: (i) "unbiased" search for the desirable factors using systemic approach combining biochemical and genetic techniques, and (ii) "express-pick" hypothesis-driven approach involving analysis of available information and creating the list of "suspects", factors that due to their known properties could possess cell-protecting properties. Below we summarize what has been done within each stage of the work, point by point.

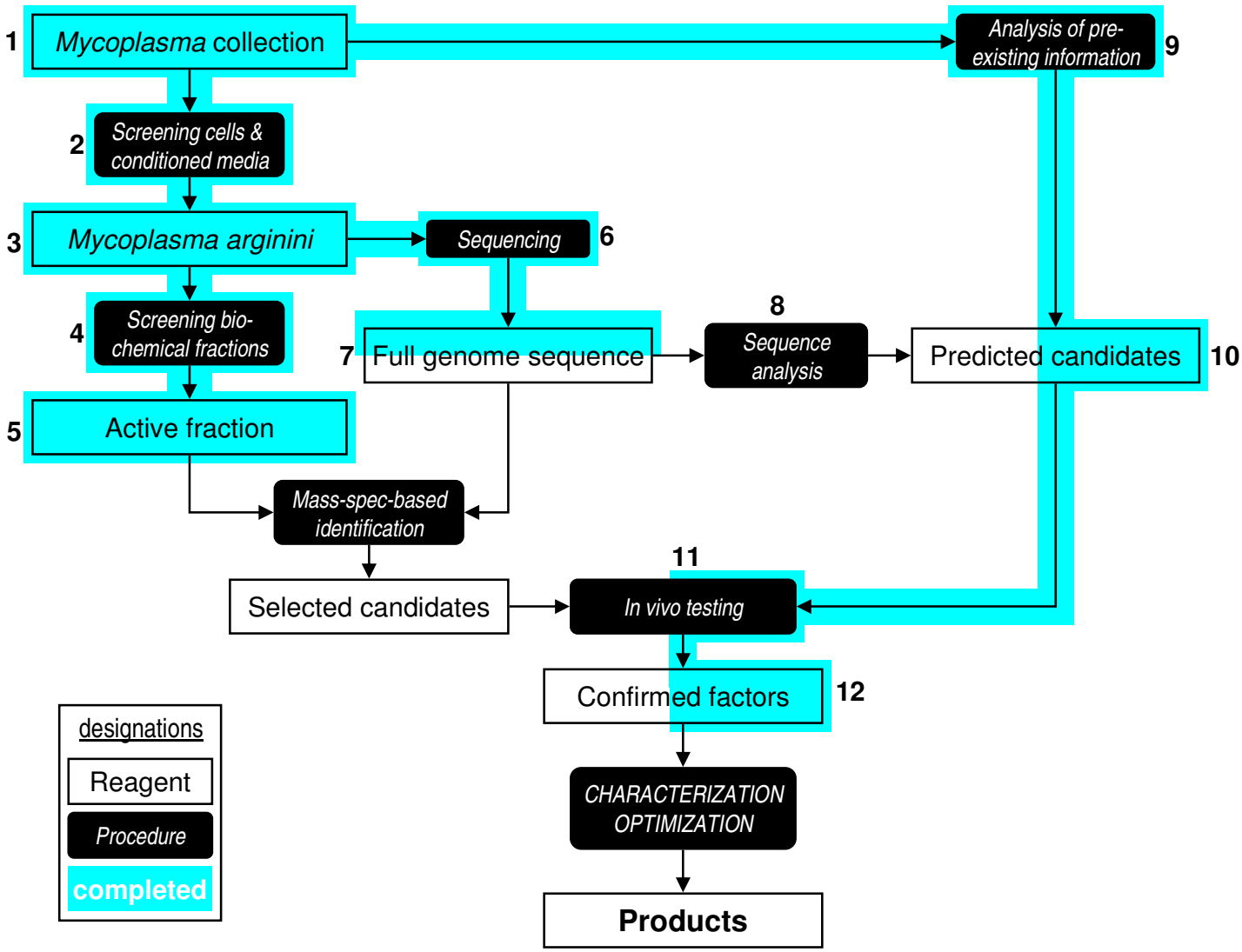


Figure 1. Outline of experimental plan and major steps of the study. Completed tasks (for Mycoplasma arginini) are highlighted. See detailed explanation in the text.

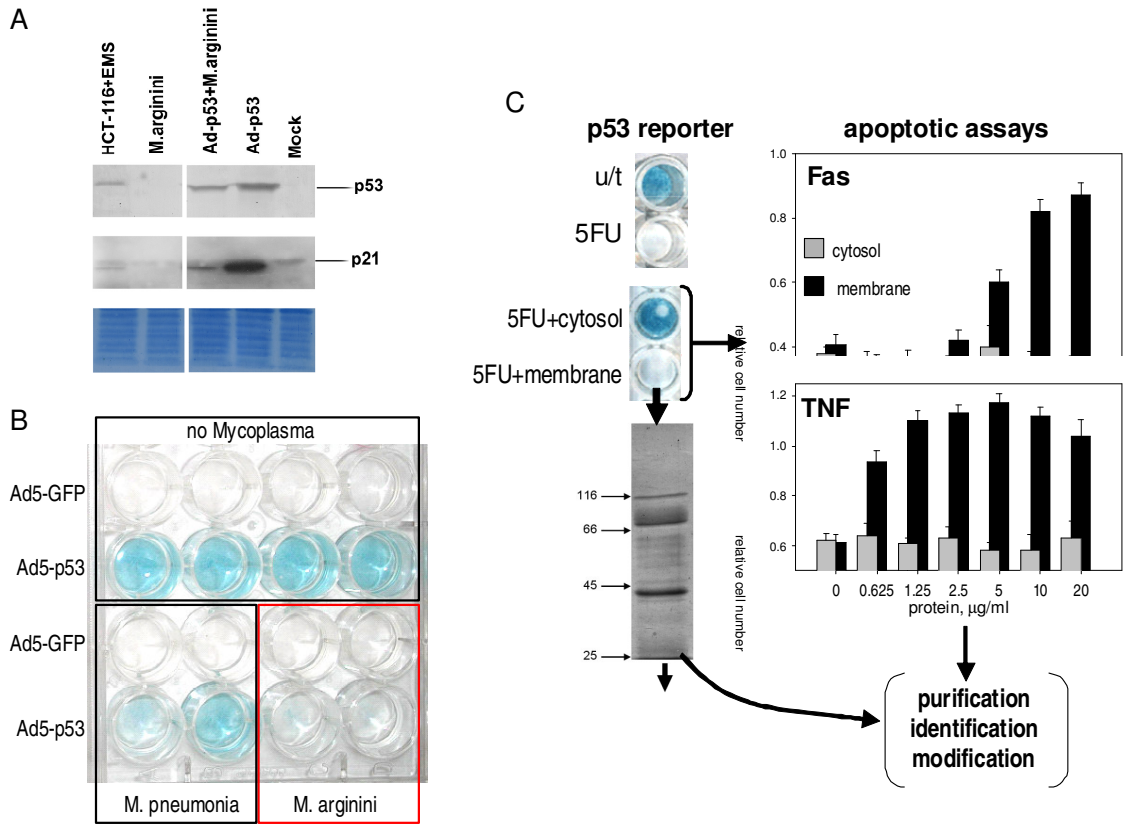
I. "Unbiased" search for the desirable factors using systemic approach combining biochemical and genetic techniques

1. ***Mycoplasma collection*** consisted of 11 species maintained in the Laboratory of Mycoplasmosis at Gamaleya Institute of Epidemiology and Microbiology, Moscow, Russia. It included most common species found in humans and other mammals residing in respiratory, urogenital and GI tracts usually as asymptomatic infection. Only a few among *Mycoplasma* species used have their genome sequenced, although small genome size (*Mycoplasma* are among the smallest bacteria with genome size around 1Mb) and its AT-richness make full sequencing a feasible task (see below). For some of the species from the collection there was experimental evidence on their association with cell protection. Moreover, infection of cell cultures with *M. arginini* was found to contribute to the multidrug resistance phenotype (A.V.G. and B.P. Kopnin, unpublished observations) that was also consistent with its putative tissue protecting activity.

2. ***Screening Mycoplasma lysates and conditioned media for pro-survival activity*** was done using a series of assays including the effects on p53 and NF-κB-mediated signaling and the ability to protect from TNF-, Fas- and staurosporin-mediated cell death. This set of assays allowed us to evaluate influence of *Mycoplasma* factors on two major death/survival pathways as well as on major types of extrinsic and intrinsic apoptosis. Effect on p53-mediated transactivation was measured in several reporter cell lines HCT116, MCF7 and ConA, carrying p53-responsive bacterial *lacZ* gene (estimated as the level of p53-inducible β-galactosidase activity); p53 was either overexpressed by adenoviral transduction or activated by treatment with DNA damaging drugs (5-fluorouracil or doxorubicin). This effect was confirmed by Western blotting analysis (Fig.2a). Protection of normal immortalized fibroblast cell line WI38 hTert from non-p53 apoptotic cell death induced by TNF, Fas or staurosporine was measured in cell survival assay quantified by methylene blue staining. All *Mycoplasma* species tested were found capable of activating NF-κB in a reporter cell line HCT116 and four species produced p53 inhibitory factors. Among Mycoplasma species tested, *M. arginini* showed most pronounced effects in the majority of assays and has become the main subject for this study.

3. ***Mycoplasma arginini***. Reports from various countries show that 10 to 87% of cell cultures are infected by mycoplasmas. The mycoplasma species infecting cell cultures have remained essentially the same over the years and *M. arginini* is among them being one of the dominant contaminants. The discovery by Ginsburg and Nicolet in 1973 [1]that *M. pulmonis* is a potent mitogen for cultured rat lymphocytes was followed by numerous reports on mycoplasma-induced nonspecific activation of immune cells, both *in vitro* and *in vivo* (for review see [2]). Mycoplasmas are mitogenic toward B- [3], T- [4], or both B and T lymphocytes. Generally, proliferation of B lymphocytes, induced by mitogenic mycoplasmas in vitro, did not require the presence of accessory cells [5]. In contrast, accessory cells were essential for *M. arginini*-induced mouse B-cell activation and were crucial for the interactions with human and mouse T cells as well [6]. Searches for putative soluble superantigens in culture supernatants of *M. pneumoniae*, *M. fermentans*, *M. hominis*, *M. penetrans* and gave no indication for the existence of MAM-like (superantigen with T-lymphocyte mitogenic activity from *M. arthritidis*) active mitogens acting on human lymphocytes [7]. It was not surprise that *M. arginini* does not have this superantigenic activity as well. Unlike *M. arthritidis* and *M. fermentans*, which trigger the production of IL-4 under certain conditions [8], other mycoplasmas (including *M. arginini*) tested so far failed to induce IL-4 in human PBMC.

Like for many others, isolated cell components of *M. arginini* were shown to synergize with IFN-γ in enhancing macrophage tumoricidal activity [6]. Peptides (2.5 to 4 kDa), probably linked to



either lipid or carbohydrate and resistant to heat and protease treatment, were apparently responsible

Figure 2. p53 inhibitory activity of *Mycoplasma arginini*. A. Effect of *M. arginini* on p53 function in HCT116 cells. Cells treated as indicated (EMS = ethylmethanesulphonate, 700µg/ml; Ad5 = infection with adenovirus 5-based vector carrying wt p53, 10 i.u. per cell) were lysed 6 hours (for EMS) or 24 hours (for Ad5) post-treatment and the amounts of p53 and p21 were determined by western immunoblotting. Mycoplasma infection does not prevent p53 synthesis but inhibits activation of p21. B. HCT116 cells carrying p53-responsive LacZ were incubated with freeze-thaw inactivated species of *Mycoplasma* followed by infection with Ad5 carrying p53 cDNA or GFP (negative control). Cells were fixed 24 hours after Ad5 infection and subjected to X-gal staining for b-galactosidase activity. *M. arginini*, but not *M. pneumonia*, effectively blocks activation of the reporter by Ad5-p53. C. M. arginini grown in cell-free medium was lysed as described in the text and individual fractions were tested for p53-inhibitory activity in HST116 cells carrying p53-responsive LacZ treated with doxorubicin (50 ng/ml, 8 hours). Protein content of the active fraction was visualized by polyacrylamide gel electrophoresis and Coomassi staining.

for the effect [9]. In agreement with these data, membrane lipoproteins of *M. arginini*, resistant to heat and proteinase K, elicited proinflammatory cytokines in human monocytes [6]. Heat-stable components from *M. arginini* also induced enhanced expression of MHC molecules on murine bone marrow cells [10]. However, the membranous elements of *M. arginini* that induced GM-CSFs were sensitive to digestion with papain, although they were thermostable [10]. It thus appears that MHC antigens and proinflammatory cytokines are elicited by membrane entities of *M. arginini* that differ from those inducing GM-CSFs. Thus, the window is still open for possibility to purify and characterize different from that of described below *M. arginini* products with potential radioprotective activity.

3.1. *Fractionation of M. arginini lysate and testing the activity of individual fractions*. Lysate of *M. arginini* was centrifuged at 40000g for 1.5 hrs. The activity was preserved in the pellet rather than in cytoplasmic, membrane-free fraction. The pellet was treated with proteinase K 0.2 mg/ml or lipoprotein lipase 0.2U/ml for 2 hours at 37^0C and tested for the ability to protect from staurosporine induced apoptosis of WI38 cells immortalized by transduction with hTert. There was no statistically significant difference between protections of enzyme-treated and untreated mycoplasma membrane-associated products from 0.5 μM staurosporine induced apoptosis.

3.2. *Active fraction* consists of water-insoluble compounds and has relatively simple protein content: SDS-PEAGE revealed several proteins in active fraction with 4 major bands with aprox. MW 47, 70, and 116 KD. (Fig. 2C). The nature of anti-apoptotic substance in this fraction is being characterized by (i) heat sensitivity, (ii) sensitivity to proteases, (iii) sensitivity to phospholipases and (iv) nucleases. Preliminary results suggest that, at least in part, anti-apoptotic activity is associated with the factor of protein nature; the identification of this factor will be possible after the completion of sequencing of *M. arginini* genome (see Fig. 2).

3.3. *Full genome sequencing*. Our efforts to obtain the partial sequence for *M. arginini* ssp. genome employ a random or "shotgun" sequencing strategy that has been the preferred approach for nearly all large-scale DNA sequencing projects to date. To create a plasmid library genomic DNA was fragmented using a computer controlled shearing device to achieve an average DNA fragment size of 2-4 kb. These fragments were size selected and cloned into gap-free cloning vector pSMART LCKan (Lucigen, Madison, WI). Transformed colonies were grown in Petri trays and individual colonies were picked into 96-well plates for growth and storage. DNA sequencing templates were prepared from the storage plates using TempliPhi cell-free amplification. Thempliphied DNA templates were sequenced on MegaBACE 4000 instrument using standard forward and reverse vector-specific primers. 1,750 plasmid clones were sequenced by SymBio Corporation (Menlo Park, CA). This number of reads resulted in 2.8 Mbp giving a total genome coverage of 3-4X. Primary assembly of the individual sequences was performed using the Sequencher software package (Ann Arbor, MI). We estimate that more than 85% of the *M. arginini* genome sequence is represented in 305 contigs. Currently, we are performing manual editing of read and contig sequences as well as manipulations with the contig layout (i.e. tearing and joining of contigs, relocating reads, etc.). This should allow us to significantly improve the quality of the assembly and the final sequence.

3.4. *Full genome sequence analysis for putative anti-apoptotic factors.* Upon completion of the assembly and editing of the genome, ORF calling and automatic annotation the *M. arginini* genome will be performed using SEED environment developed by Fellowship for Interpretation of Genomes (FIG). SEED development is at the heart of National Microbial Pathogen Data Resource Center and is supported by a major NIH grant. Installation and support of the system will be provided by FIG, which has a long history of collaboration with CBL.

SEED genome analysis system is an advanced comparative genomics tool capable of the advanced annotation of genome content and creation of a high order of integration of genome information - functional reconstruction. This integration put gene assignments in a context of whole-organism metabolism thus significantly reducing error rate in prediction of gene functions. SEED system utilizes data from nearly 500 sequenced genomes and is ideally suited for the type of context analysis necessary to pinpoint bacterial genes, which can be involved into the regulation of apoptosis in mammals. SEED and its predecessors WIT and ERGO were part of about a dozen of high visibility publications describing sequencing and annotation of complete or gapped genomes.

Based on our prior experience with low-coverage genomes sequencing we expect to detect more then 95% of *mycoplasma* genes (some of which will be split by gaps or truncated) and assign functions to about 70% of putative ORFs. We expect to identify a vast majority of the metabolic pathways and reconstruct a functional net of the organism.

The final step of the genome analysis, the identification of putative anti-apoptotic factors from *M. arginini* will be done by projection of the mycoplasma genes on known metabolic and regulatory pathways, which may be involved in apoptosis and by finding bacterial homologs of human genes, which can be implicated in the process of interest – regulation of apoptosis.

II. Literature-based prediction of *Mycoplasma* factors with radioprotective purposes and their testing

1. ***Analysis of pre-existing information***. We have performed the analysis of information regarding factors produced by Mycoplasma, which might be involved in control of host mechanisms of stress response. It is increasingly recognized that cytokine induction via activation of NF-κB pathway is a major virulence mechanism of Mycoplasma [2, 6, 11-13]. The induced cytokines have a wide range of effects on the eukaryotic host cell and are recognized as important mediators of tissue pathology in infectious diseases. It appears that although mycoplasmas circumvent phagocytosis, they interact with mononuclear and polymorphonuclear phagocytes stimulating the synthesis of cytokines with proinflammatory action. These immunomodulatory influences depend on both the immune cells and the *Mycoplasma spp.* involved. Macrophage-mediated cytolysis by whole cells of *M. orale* was first described by Lowenstein et al. at 1983 [14]. Cytolysis of the neoplastic cells was obtained even with macrophages from the lipopolysaccharide (LPS)-unresponsive C3H/HeJ mice, suggesting that the mechanism of activation is different from that of LPS [15]. Since then over 20 *Mycoplasma* species have been shown to activate monocytes, macrophages, and brain astrocytes and induce secretion of the proinflammatory cytokines tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, chemokines, such as IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1α), granulocyte-monocyte colony stimulating factors (GM-CSFs), as well as prostaglandins and nitric oxide [2]. More recent observations suggest that the mechanisms underlying macrophage activation by whole cells are in many cases identical to those employed by their purified membrane lipoproteins, supporting the notion that lipoproteins are the principle component of intact mycoplasmas activating monocytes/macrophages and playing an important role in the inflammatory response during infection [12, 16-21].

2. ***Predicted candidates***. Immunomodulatory lipoproteins include, for instance, macrophage-activating lipoprotein 2 (MALP-2) of *Mycoplasma fermentans* [22], VlpA and VlpC of *M. hyorhinis* [18], and FSL-1 from *M. salivarium* [12]. Lipoproteins are found in the cytoplasmic membrane and in the outer membrane of almost all Gram-positive and Gram-negative bacteria[23-26]. All membrane-anchored bacterial lipoproteins contain a lipoylated amino-terminal cysteinyl residue which, in some cases, is *N*-acylated [27]. Lipoproteins are extremely abundant in the cell membrane of mycoplasmas. In *M. pneumoniae*, for example, of an estimated number of 150 membrane proteins, 46 open reading frames encoding putative lipoprotein genes have been identified. There are at least 35 acylated proteins in *M. gallisepticum*. Chemical analyses of mycoplasmal lipoproteins have revealed that their lipoylation mechanism is similar to that of Gram-negative and Gram-positive bacteria [27]. However, in many mycoplasmas, the lipoproteins are not *N*-acylated, nor has an *N*-acyltransferase gene been found in the genome . The first reports on the cytokine-inducing ability of mycoplasmal lipoproteins showed that a lipoprotein from *M. fermentans* or *M.*

arginini is capable of stimulating the release of proinflammatory from human peripheral blood monocytes in a dose-dependent manner. Comparison of the effects of intact lipoproteins with those of proteinase-K-treated lipoproteins reveals that the lipoylated amino terminus is responsible for the immunostimulating properties of the lipoproteins. However, it is not certain whether all naturally occurring mycoplasmal membrane lipoproteins are potent macrophage activators. The importance of the lipid residue has been emphasized by the isolation and characterization of naturally occurring lipopeptides with macrophage-activating potential from several mycoplasmas.

Name	**Structure**
Confirmed for NF-κB activation	
Pam2	Pam2C-SKKKK
M. salivarium (FSL-1)	Pam2C-GDPKHPKSF
M. fermentans (MALP-2)	Pam2C-GNNDESNISFKEK
M. mycoides	Pam2C-GDKTPSTKSAGKVENK
M.gallisepticum (p47)	Pam2C-GAASSLTYESSVQLVVSDNSS
M. penetrans (p35-2)	Pam2C-GSPLSFESSVQLIVSDNSS
M. penetrans (p35)	Pam2C-SSTSENNGNGNGNGGTD
M. hyorhinis	Pam2C-GQTDNNSSQSQQPGSGTTNT
Mycobacterium tuberculosis(p19)	Pam3C-SSNKSTTGSGETTTAAGT
M. pulmonis	Pam2C-AQNPNKTNSNLDSSK
M. arthritidis (Maa2)	Pam2C-DNEEKPTPEQD
Pam3	Pam3C-SKKKK
E. coli (murein lipoprotein)	Pam3C-SNNA
Neisseria gonorrhoeae (CCP)	Pam3C-GGQEKSAAG
*Neisseria gonorrhoeae (*Cytochrome *c′*)	Pam3C-GNGGAPAQPKG
NOT Confirmed for NF-κB activation	
M. agalactiae	Pam2C-GDKYFKETE
Thioredoxin	Pam2C-GPCPGCPPC
Thioredoxin-2	Pam2C-PPCPGCPPC
M.arginini	Pam2C-GETDKEGKIIRIFDNSF
Porphyromonas gingivalis (fibrilin)	Pam2C-NKDNEAEPVTEGNAT
Porphyromonas gingivalis(P75)	Pam2C-SKEGNGPDPDNAAKS
Staphylococcus (PSM1)	Pam3C-ADVIAKIVEIVKGLIDQFTQK
Staphylococcus (PSM4)	Pam3C-SIVSTIIEVVKTIVDIVKKFKK
H. pilori	Pam3C-SNYAKKVVKQKNHVYTPVY
Mycobacterium tuberculosis(MPT83)	Pam3C-SSTKPVSQDTSPKPATSPAAPVTTA
Neisseria meningitides	Pam3C-SGALAATSDDDVKKAATVAIVA
Neisseria meningitides (LP2086)	Pam3C-SSGGGGVAADIGAGLADALTAP
Neisseria meningitides (nitrite reductase)	Pam3C-GGEPAAQAPAETPAAAAEAAS
T. pallidum 47-kDa major lipoprotein	Pam3C-GSSHH
B. burgdorferi OspA	Pam3C-KQNVS
B. burgdorferi OspC	Pam3C-NNSGK

After the completion of full genome sequencing, bioinformatics will likely indicate additional candidates for "express-pick" analysis (i.e., proteins sharing homologies with known factors involved in regulation of cell death). Their corresponding genes will be synthesized to convert *Mycoplasma* sequences into regular genetic code, cloned in E. coli expression inducible vectors in frame with poly-histidine tags for affinity purification of the encoded proteins. After induction, Mycoplasma proteins will be purified from the bacterial lysates under the conditions eliminating the contamination with endotoxin and subjected to further analysis. However, even before full sequence of *Mycoplasma arginini* we found that there are several potential genes, which may code for lipoproteins and Ag243-5 was among them.

3. *CBLB601*. So far, we have tested several predicted lipopeptide candidates, among them e.g. FSL-1, for radioprotective properties in vivo. However, in the majority of cases, the efficacy was limited by the solubility problems (we decided not to test DMSO solutions because of their limited therapeutic potential). Nevertheless, among the candidates tested, one has successfully passed through the in vivo testing stage. This is R-PAM$_2$C-SKKKK - synthetic lipopetide (R-Pam2-CSK$_4$). It was chosen based upon the fact it has the same NF-kB activation property as naturally existing ones such as MALP-2 and FSL-1 from two different *Mycoplasma spp*, and notion that *R*- (but not *S*-counterpart) isomer exhibits biological activities. Further on, R-Pam2-CSK$_4$ will be referred as CBLB601 as it was designated to serve the basis for generation and development of a novel group of radioprotectors, *Protectans of the 600 series.* Interesting that natural homologue of CBLB601 – MALP-2 – was found to be very potent immunomoduloator with strong adjuvant activity. Moreover, MALP-2, induces in vitro tumoricidal activity of macrophages. It is also highly active in vivo as it induces leukocyte infiltration after intraperitoneal administration in mice or after intratracheal administration in rats. In this system, it reduces formation of metastases in the lung. MALP-2 was shown to exhibit adjuvant properties when applied intranasally. Studies in BALB/c mice bearing methyl cholanthrene-induced fibrosarcoma showed strong haemorrhagic tumor necrosis with complete healing 10 days after MALP-2 application. Last year phase I/II clinical trials for patients with resected pancreatic carcinoma have been started with intratumoral injection of MALP-2 in the remaining tumor bed.

3.1. *Determination of MTD (maximal tolerable dose).* Since we considered testing of CBLB601 efficacy in the regimen of single dose total body irradiation (TBI), we first embarked upon determination of maximal tolerable dose in a single dose administration regime. ICR mice were i.p. injected with increasing doses of CBLB601 ranging from 0.3 to 100 ug/mouse. No mortality was observed at these doses. However, at 100 ug/mouse the signs of morbidity, like slow motion and scruffy fur became evident. Thus, we have determined the MTD as 30 ug/mouse.

3.2. *Determination of optimal radioprotective dose.* ICR mice were injected with different CBLB601 doses (from 1 to 30 ug/mouse) 24 hrs prior to 10Gy TBI and their survival was monitored for 30 days. As evident from Figure 3, the best protective dose was 3 ug/mouse supporting survival of 100% of irradiated mice. Almost similar efficacy was reached with 1 and 10 ug/mouse doses, which rescued 90% of mice. In contrast, higher CBLB601 doses (20 and 30 ug/mouse) led to accelerated mouse mortality when administered in combination with γ-irradiation. The observed accelerated death may probably stem form a combined toxicity of both treatments which both result in cytokine outburst. Further experiments are needed to determine maximal nontoxic dose which my be safely administered in combination with TBI with better precision. *Currently optimal protective dose of CBLB601 is determined as 3 ug/mouse.* We concluded that *CBLB601 conferes radioprotection at the doses that 10-30-fold lower than MTD.*

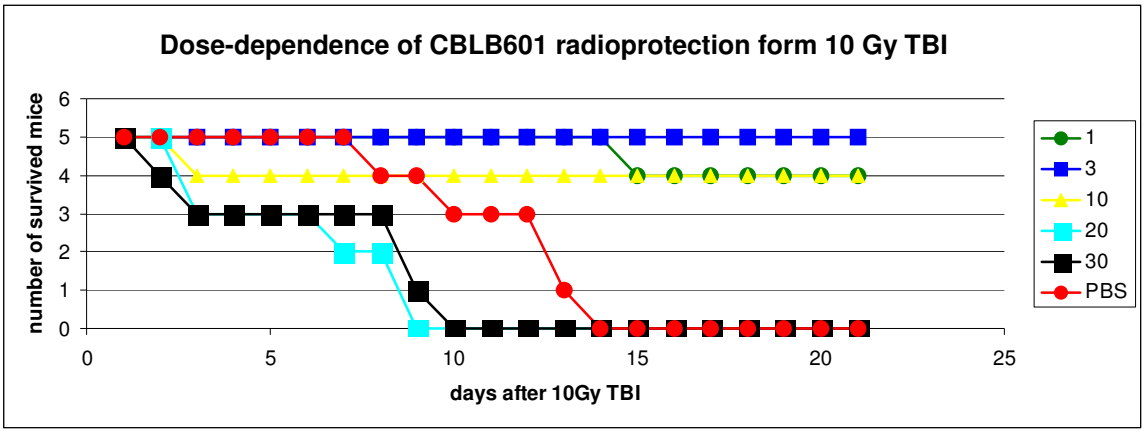


Figure 3. Determination of the optimal radioprotective dose of CBLB601.

3.3. Determination of optimal CBLB601 administration schedule. Establishment of the optimal time schedule for CBLB601 administration is the key point for comprehensive evaluation of radioprotective efficacy of the substance. 3 ug of CBLB601 were intraperitoneally injected into ICR mice at different time points prior to 10Gy TBI. The injection time varied from 30 minutes to 96 hrs prior to irradiation. The results of these experiments are summarized in Figure 4. It is clear that among the conditions tested, injection of CBLB601 24 hrs before irradiation yields the best radioprotections, although administration at -48 hrs was also effective to a certain degree. Future experiments will be dedicated to fine tuning the activity window. For this, more efficacy studies with CBLB601 delivered between –6 and –48 hrs with smaller intervals should be performed. Of extreme importance will be elucidation of radioprotective potential of CBLB601 when injected at various time points after irradiation. *According to current data, the optimal time point for CBLB601 administration was defined as –24 hrs prior to TBI.* Thus, for the intermediate evaluation of CBLB601 DMF, the drug will be administered according to the regimen, which was currently found optimal: 3 ug/mouse 24 hrs prior to irradiation.

3.4. *Developing cell-based system for CBLB601 activity testing in vitro.* To be able to test more radioprotectants the Protectans600 series and to further investigate biochemical properties of CBLB601, we have developed fast, reliable, and quantitative *in vitro* cell-based readout system. The system is based on a pair of isogenic cell lines either expressing or not expressing TLR2/TLR6 heterodimer, which is the ligand for Pam2-containing lipopeptides. Both cell lines contain NFkB-driven LacZ reporter transgene. Following addition of the test substances, the cells are incubated at 37C overnight and then LacZ activity is evaluated by ONPG staining. When TLR2/TLR6 expressing resporter cells are treated with increasing concentration of CBLB601, it triggers TLR2/TLR6-dependent and dose-dependent LacZ activation (Figure 5). Concentrations of CBLB601 as low as 5 pM were already effective in eliciting TLR2/TLR6-dependent reporter activity. In contrast, none of CBLB601 concentrations were effective in activation of the analogous reporter in TLR-null isogenic reporter cells (not shown), thus, confirming the ability of CBLB601 to activate TLR2/TLR6 heterodimers.

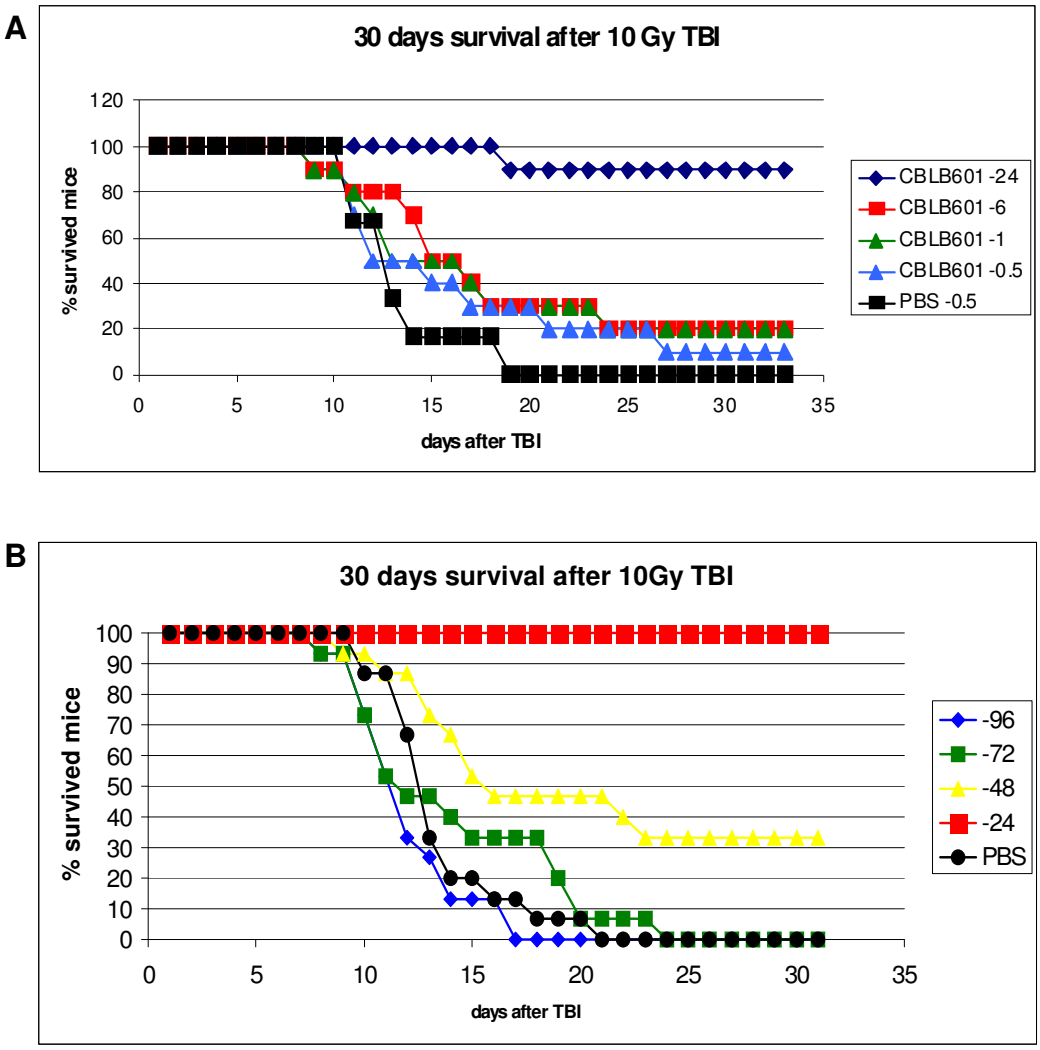


Figure 4. Determination of the optimal time frame for administration of CBLB601 to confer protection from 10Gy total body irradiation

3.5. Testing of the influence of storage conditions on radioprotective activity of CBLB601. The developed in vitro assay was used to evaluate whether long-term storage of CBLB601 as water solution will impair its biological activity. Different PBS solutions of the same batch of CBLB601, which were stored either at +4C or at –20C for different time intervals were added to TLR2/TLR6-NfkBLacZ cells and their relative activity was compared (judged by dose response reporter activity curves). Figure 5 clearly demonstrates that CBLB601 was remained fully active regardless of the storage conditions

explored. We next analyzed, whether these same preparations of CBLB601 demonstrate similar radioprotective properties in vivo and found that also in vivo radioprotective activity of CBLB601 PBS solutions was not impaired by a prolonged storage (Figure 6). In the future, we plan a more systematic assessment of storage stability and other important characteristics of the compound.

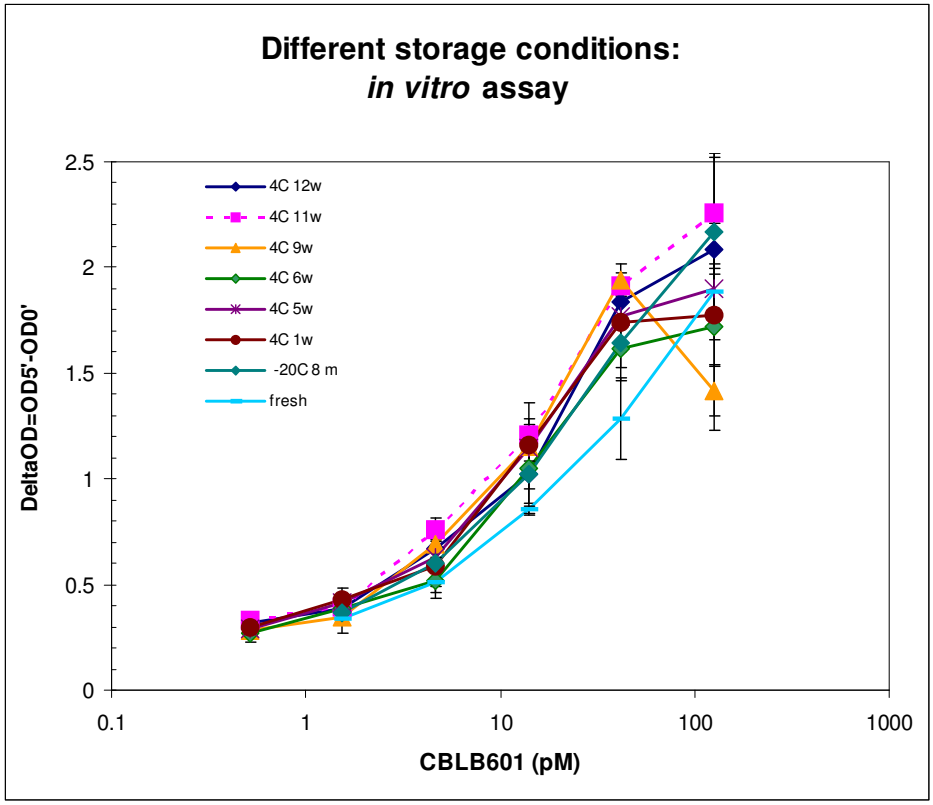


Figure 5. Assessment of NFkB inducing activity CBLB601 solution stored in different conditions in TLR2/TLR6 in vitro assay.

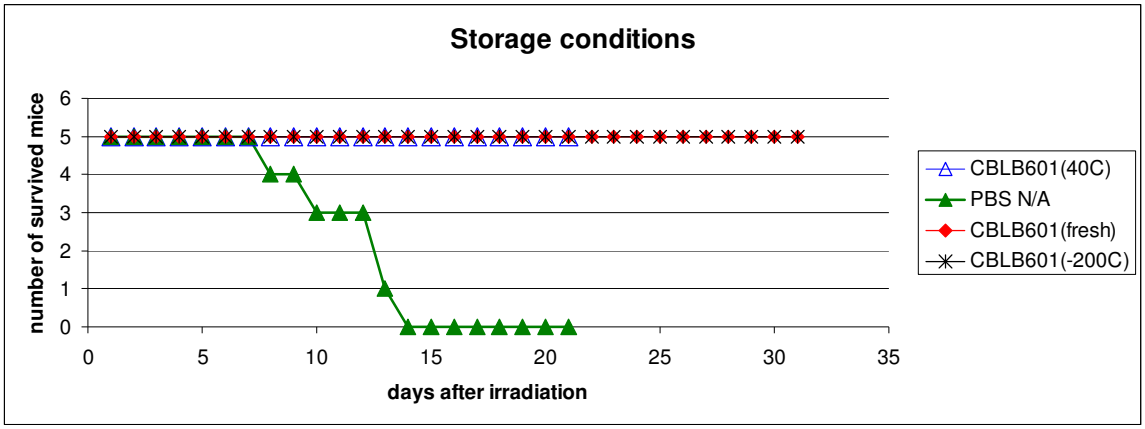
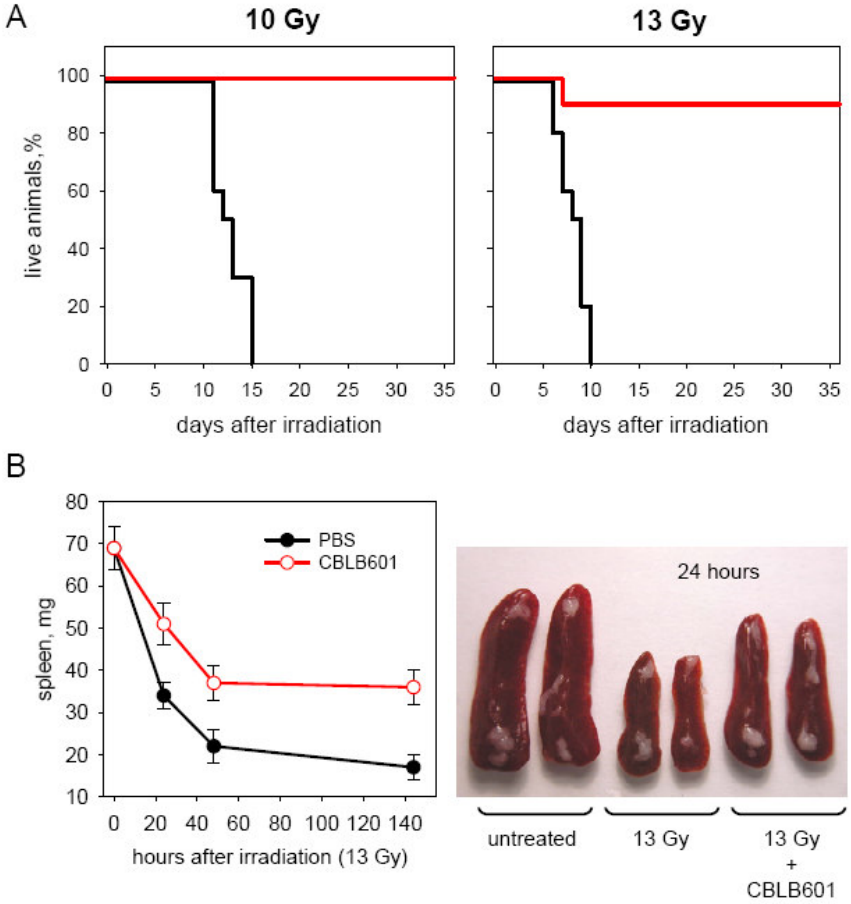


Figure 6. Comparison of in vivo radiprotective properties of differently stored CBLB601

3.6. *CBLB601 protects gastrointestinal tract and lymphoid organs from supralethal doses of TBI.* In some of the early calibration experiments performed with CBLB601 in NIH Swiss mice, we were occasionally capable to reach almost complete protection not only from the 10Gy (hematopoietic syndrome dose) but also from 13Gy (gastrointestinal syndrome dose) of TBI. This indicates that further optimization of administration conditions may yield even more powerful radioprotection than currently established for this compound (Figure 7A). Importantly, it caused effective protection of spleen and thymus from 13 Gy of radiation, which has been never reported for any other protectant. Figure 7B shows quantitation of the dynamics of spleen size after 13 Gy of total body gamma radiation as well as the gross morphology of spleens from untreated and irradiated animals that received or did not receive Protectan 601. The analysis of mice that survived 30 days post 13 Gy demonstrated normal size of thymus suggesting that thymus recovery occurs much more effectively that in similar animals that were rescued by flagellin derivative (data not shown). It is noteworthy that radiation response of splenocytes and thymocytes is mostly p53-dependent but that p53 inhibition is incapable of protecting epithelium of small intestine, which can be well rescued by NF-kB activators (flagellin). The ability of CBLB601 to effectively protect splenocytes, to support fast recovery of the thymus and at the same time to protect GI tract from radiation damage suggests



that this factor can simultaneously target p53 and NF-kB, the hypothesis that is currently under testing.

Figure 7. Radioprotective effect of CBLB601 in NIH Swiss mice. A. Dynamics of radiation-induced death following indicated single doses of total body gamma radiation (^{137}Cs source, 400 cGy per minute). B. Alterations in the size of mouse spleens following 13 Gy of total body gamma irradiation with and without pre-treatment with Protectan 601 (i.p. injection of 3μg/mouse, 30 min. prior to irradiation).

3.7. *Importance of TLR2/TLR6 activation for radioprotection by lipopeptides.* CBLB601 is a synthetic lipopetide R-PAM$_2$C-SKKKK (Pam2), a derivative of naturally existing substances – bacterial lipoproteins (Lpps). Lpps are observed in cell walls of a variety of microorganisms. Host immune cells recognize the specific patterns of Lpps through the association of TLR2 with other TLRs, specifically TLR6 or TLR1. TLR2 heterodimers mediate the response to the most diverse set of molecular structures, including peptidoglycan, lipoteichoic acid, lipoarabinomanan, bacterial Lpps, and also some LPS variants from Gram-negative bacteria, yeast, spirochetes and fungi. It was previously demonstrated that the diacyl-Lpp MALP2 from *M. fermentas* (as well from another *Mycoplasma ssp.*) requires TLR2/ TLR6 heterodimer for signaling, whereas the triacylated synthetic compound N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl -(R)-cysteine (Pam3)-CSK4 is able to induce activation of innate immune cells independently of TLR6 and mainly through TLR2/TLR1 heteromers. These findings and additional investigations using various di- or tri-acylated Lpp have led to the current model: triacylated Lpp signal through TLR2/TLR1 heteromers, whereas diacylated Lpp signal through TLR2/TLR6 heteromers.

Hence, to clarify which of the TLR2 co-receptor (or both) is indeed involved in radioprotection it was important to check experimentally which Lpp (having knowledge about TLR1/6 specificity form *in vitro* experiments) would be effective radioprotector *in vivo*. To address this issue, we have synthesized a peptide identical to the one present in CBLB601, CSKKKK, which was linked not to Pam2, as in CBLB601, but to Pam3. To test whether indeed Pam3-CSK4 does not activated TLR2/TLR6 heteromers, we assessed its NFkB activation potential in in our TLR2/TLR6-dependent reporter cells. The results shown in Figure 8 clearly demonstrate that Pam3-CSK$_4$ was incapable of NFkB activation in TLR2/TLR6 receptor context.

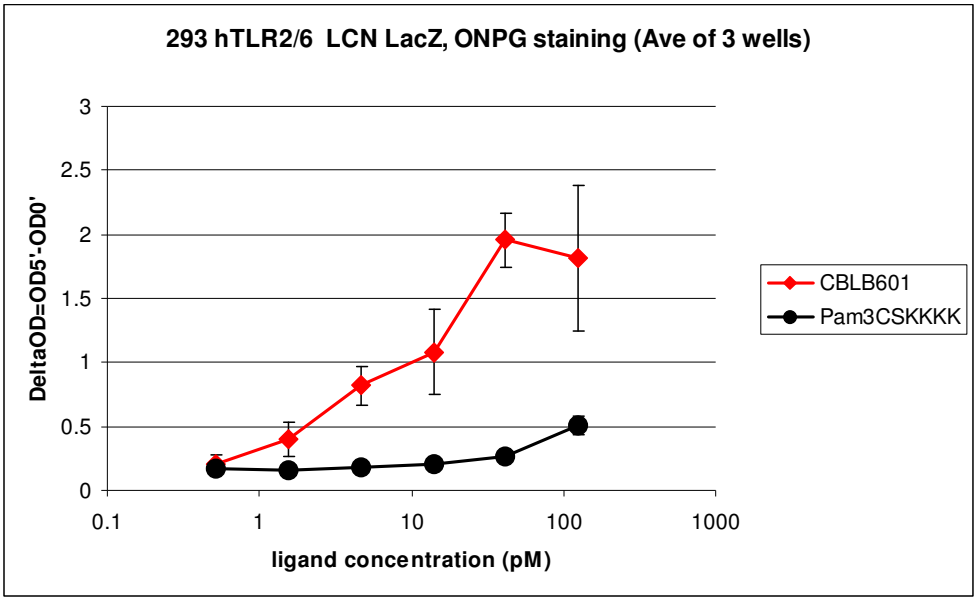


Figure 8. Assessment of R-Pam2-CSK$_4$ (CBLB601) and Pam3-CSK$_4$ NFkB activating potential in TLR2/TLR6 reporter cells

Next, we tested radioprotective properties of Pam3-CSK$_4$ in vivo using CBLB601 and PBS as positive and negative controls, respectively. Whereas in this experiment CBLB601 was capable of rescuing

mice from 13Gy TBI (Figure 9), no 30-day radioprotection was elicited by Pam3-CSK_4, however a certain shift in the mortality schedule was observed. This shift, however, indicates a lack of the ability of Pam3-CSK_4 to protect hematopoietic system. Thus, we concluded that triggering of specifically TLR2/TLR6 dimers confers radioprotection the best. This initial finding will be explored further in a more systematic experimental set-up. However, based on this finding, we are already planning to concentrate our effort on additional TLR2/TLR6 ligands and also considering generation of artificial ones of non-bacterial origin (e.g., small molecules or agonistic antibodies).

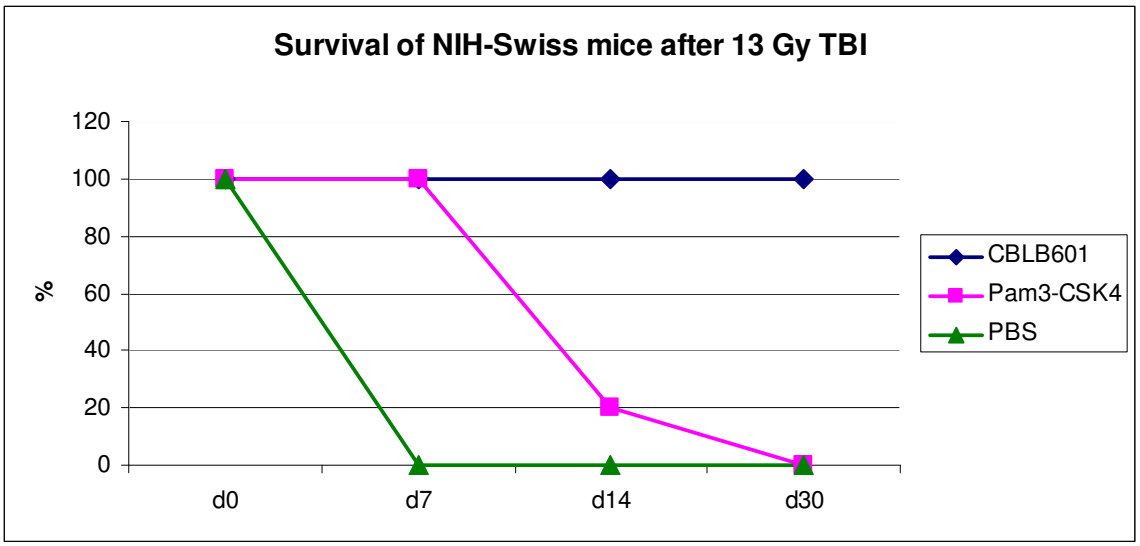


Figure 9. Comparison of radioprotective properties of R-Pam2-CSK_4 (CBLB601) and Pam3-CSK_4 in vivo in NIH-Swiss mice received 13Gy TBI. The compounds were injected i.p. 30 minutes prior to irradiation, 3 ug/mouse (n=10).

Publications and technical reports supported under this grant or contract

Papers published in peer-reviewed journals – *none*

Papers published in non-peer-reviewed journals or in conference proceedings - none

Papers presented at meetings, but not published in conference proceedings - none

Manuscripts submitted, but not published – *none*

Technical reports submitted to ARO – interim report, submitted March 2004

List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project

Andrei Gudkov, Ph.D., D.Sci., Chairman of the Department of Molecular Biology of the Lerner Institute at the Cleveland Clinic Foundation, founder and SCO of CBL, is a scientist with over 25 years of experience in biomedical research, author of >120 research papers and inventor on 21 issued US biotechnology patents. The current proposal is based on his innovative concept of suppression of apoptosis in order to reduce side effects of anticancer therapy. Iin 1999 he defined p53 as a major determinant of cancer treatment side effects and suggested this protein as a target for therapeutic suppression. Another conceptual foundation of the proposal, microbial proteins as a source of apoptosis-modulating compounds, has also originated from Dr. Gudkov's research.

Michael Fonstein, Ph.D. pioneered the field of microbial genomics performing long-range physical mapping of *E. coli* and *C. glutamicum* in mid eighties. Later he supervised a dozen genome sequencing and annotation projects aimed at bacterial pathogens at the Integrated Genomics, Inc. (IG). Specific research projects supervised by Dr. Fonstein are highly relevant to the current proposal, including comparative genomics of *B. cereus* and *B. anthracis*, as well as functional reconstruction of metabolism of numerous sequenced microorganisms.

Pavel Komarov, Ph.D., Director of HTS core of CBL, has extensive experience in molecular biology of cancer, high throughput screening, drug discovery and hit-to-lead optimization processes. He has more than 40 publications in peer-reviewed scientific journals and two patents related to the proposal subjects. He has a unique blend of extensive experience with both HTS and mechanism of action of radioprotectors. In collaboration with Dr. Gudkov, Dr. Komarov pioneered isolation of the first small molecule p53 inhibitor pifitrin-alpha.

Eugenia Strom, Ph.D. Dr. Strom, scientists of CBL, with strong expertise in microbiology, molecular and cellular biology. She holds Ph.D. degree in microbiology from Moscow State University (Moscow, Russia). She worked in CBL since 2003 as Research Scientist where she is primarily responsible for identification and characterization of radioprotective and apoptosis suppressive role of microbial and tumor proteins.

Alexander Shakhov, Ph.D. Dr. Shakhov is an expert in molecular and cellular biology with more than 25 years of experience. During last 7 years he worked in National Cancer Institute (NCI), where he was involved in gene expression which are under TNF/LT control. Prior to this, Dr. Shakhov worked in Ludwig Institute for Cancer Research and Institute of Toxicology (Switzerland), where he leaded multiple projects of gene regulation. He was involved in over 50 publications. He joined CBL as a Project Leader in 2004, where he is responsible for development of new generation of radioprotectors.

Elena Feinstein, M.D., Ph.D. Dr. Feinstein is CBL's Vice President for R&D. Dr. Feinstein has a unique expertise in molecular, cellular, cancer and system biology, and functional genomics. While working in Weizmann Institute of Science, she pioneered application of new functional genomics technologies to discovery of new cancer-related genes. During the last five years she worked as Director of R&D at Quark Biotech, Inc. (QBI)) where she was primary responsible for establishment of R&D programs in cancer, fibrosis, bone disorder, diabetes type II and other disease areas. She has rich experience in gene expression and functional profiling with specific focus in oncology and metabolic disease models. At CBL she supervises internal R&D activities as well as collaborative research projects with academic partners, including work with Dr. Gudkov and other investigators at CCF.

Report of Inventions (by title only) - attached

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