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REVIEW PAPER

Searching for synergy *in silico*, *in vitro* and *in vivo*

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Summary In this paper, I examine the role of the idea of synergy in life science research using examples in the fields of pharmacology/toxicology, molecular genetics and development, biochemistry, ecology and metabolic engineering. The research shows that synergy exhibits scale invariance. Small molecules act synergistically in the activation of single receptor molecules. Proteins function synergistically in development, metabolism and signaling. Synergy was found in the interaction between communities of organisms. Synergy manifests itself quantitatively or qualitatively: synergistic effects can be smaller or larger or they can be entirely different from what was expected. There is no single mathematical model that can be used uniformly to detect and quantify synergy. Synergy provides benefits for human health, wellbeing and economy. Synergy has explanatory and heuristic value in our quest to understand the function of and in designing complex biological systems.

Contents

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1. Introduction

What is synergy? Where should we look for it? Will we recognize it when we find it? In this paper, I will present illustrative examples of research in the life sciences where the discovery of synergy itself was purposely pursued or the idea of synergy was used for conceptualizing unexpected experimental results. This paper is not a comprehensive review of how synergy is defined and applied in any particular field of research; nor are the examples presented here necessarily representative of any given field of inquiry. I rather subjectively chose instructive examples from the scientific literature mainly in the fields of pharmacology and molecular genetics including only a few of examples from other fields in the life sciences. I predominately selected studies that either purposely searched for synergy or when a particularly striking instance of synergy was encountered possibly unexpectedly. Furthermore, I chose studies that made use of model systems or experimental approaches that in my view might be usefully applied to more than one field of research. I will begin by looking at the different connotations of the word synergy in the English language at different times and in different fields.

The word *synergy* is derived from the Greek word συνέργεια (*synergia*) from, συνεργός (*synergos*), meaning 'working together' [1]. According to the Oxford English Dictionary (OED), it is in this literal sense that the word entered the written English language in the 17th century and the European medical literature in the 19th century. The OED lists three definitions of synergy which contain cross references to synergism [2]: (1) "Joint action, cooperation; *esp.* (*Theol.*) cooperation between human will and divine grace in the work of regeneration (*cf.* synergism *n.* 1, synergist *n.* 1)" (used first in 1632), (2a) "Combined and coordinated action (by muscles, other organs, types of cell, *etc.*) in the performance of a specific movement or function; an instance of this; = synergism *n.* 2a" (used first in 1820), (2b) "Chiefly *Pharmacol.* = synergism *n.* 2b." "Interaction (between substances, processes, *etc.*) that produces a heightened effect, spec. a combined effect which is greater than additive; = synergy n. 2b." (synergism and synergy used first in this sense in 1904 and 1917, respectively) and (3) "Any interaction or cooperation which is mutually reinforcing; a dynamic, productive, or profitable affinity, association, or link" (used first in this sense in the 1950s).

Consistent with the OED, an analysis of the use of the word *synergy* based on the Google collection of digitized books indicates that the first documented use of this word in the English language was as a synonym of "co-operation" [3,4] and that it was used in this sense also in the European medical literature at the beginning of the 19th century. For example in the Elementa physiologiae ad usum praelectionum academicarum, Lorenzo Martini used the term "synergia, seu adsociatio est plurium partium ad eamdem obeundam functionem conspiratio" [5]. Martini gave as an example of synergy the liver and stomach, two organs that work together with the same purpose, namely digestion. In the life sciences, definitions 2a and 2b, underpin roughly the work in systems-oriented and molecular life science, respectively. Neuroscientists and engineers studying motor control in the nervous system and its application in robotics often use the plural of *synergy*, which is *synergies*. In this line of work, muscle synergies are defined as coordinated activations of a group of muscles [6–8]. The muscle synergy hypothesis states that the brain combines a set of synergies in a task-dependent fashion in order to generate the muscle contractions that lead to the desired movement [6,8]. Another definition of synergy in motor control was given by Latash and Anson [9] stating "... that a *synergy* represents an organization of elemental variables (...) that stabilizes an important performance variable. *Elemental variables* are the smallest sensible variables that can be used to describe a system of interest at a selected level of analysis." [9]

The various connotations of *synergy* are referenced in the online Wikipedia: "Synergy is the interaction of multiple elements in a system to produce an effect different from or greater than the sum of their individual effects." This definition encompasses both the original 17th century notion of synergy as collaboration ("interaction ... to produce") and the 19th century and 20th century idea of quantitatively ("greater than ...") or qualitatively ("different from ...") increased or changed effects.

In the English language, the use of the word synergy has been increasing since the mid 1800s with considerable acceleration beginning in the 1960s (Fig. 1). In July 2014, using the

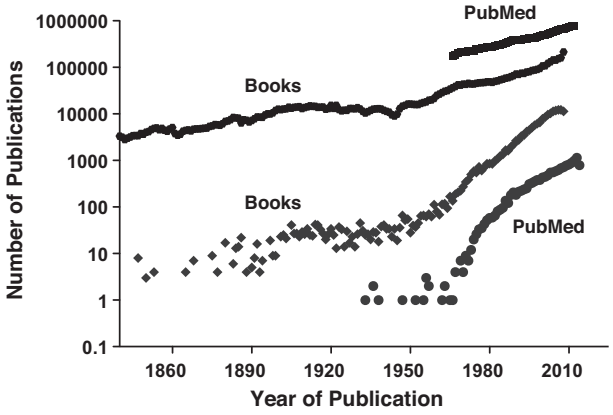

Figure 1 Word usage graph for "synergy". PubMed and Google Ngram were searched for the words "synergy" (case insensitive) to determine the yearly frequency of occurrence of the word in books and PubMed citations (title and/or abstract). Top to bottom: the first two lines are counts of the total number of PubMed citations and books in the Google Ngram data set, respectively. The filled diamonds indicate the number of books and the filled circles the number of PubMed citations containing the word "synergy".

search term "synergy OR synergism" returned more than 77,000 entries in PubMed [10], a MEDLINE database of references and abstracts on life sciences and biomedical topics. The earliest publication is from 1927 [11]. Almost 80% of these publications concern the field of pharmacology; approximately 12% are related to the field of genetics (about 10% are related to both fields), and less than 4% to biochemistry. The following presentation of published work illustrating the role of the concept of synergy in life science research is loosely grouped under the headings of pharmacology, molecular genetics and development, biochemistry, environmental toxicology, ecology, and synthetic biology and metabolic engineering. The headings reflect the main thrust of the work and allow for quick orientation but are not intended as classification. I did not include work related to muscle synergies and point the interested reader to references [6—9].

2. Synergy in pharmacology

The search for synergy in the field of pharmacology concerns the elucidation and quantification of the action of drugs applied in combination. Two or more drugs administered together are considered to act synergistically if the observed effect is greater than the effect expected from the effect of each drug administered alone [12]. Drugs are said to act antagonistically, if the combined effect is less than that observed upon separate administration of each drug. Thus, synergy is defined as being present when the observed effect exceeds the expectation. One then comes to the curious conclusion that synergy is based on a lack of knowledge [12—15]. For almost a century (throughout various times), an acrimonious debate has raged in the scientific literature concerning the correct definition, terminology, mathematical models and statistical testing procedures for determining synergy of drug combinations [12—38]. Researchers have commonly used three models to quantify synergistic effects. These models are referred to as Loewe Additivity [12,39], Bliss Independence [40], and excess over Highest Single Agent (HSA; attributed by Berenbaum [15] to Gaddum [41]). Loewe additivity underpins both the use of isobolographic analysis [13,15,19,42] and the so-called combination index (CI) published in 1984 by Chou and Talalay [17]. The history of the development of these models was comprehensively reviewed by Berenbaum [15] and recently by Geary [18]. In spite of these theoretical difficulties, the allure of combination therapies and drugs keeps increasing with the U.S. Food and Drug Administration (FDA) taking note of the benefits and risks of combination drugs [43].

In the modern era, synergistic effects of drugs were first investigated and then applied to treat microbial diseases with drug combinations [42,44—49]. A common rationale for anti-microbial combination therapy is to avoid or overcome microbial drug resistance [50]. Levin and Harris [48] listed eight indications for antimicrobial combination therapy and noted that synergy was only one of those eight indications. The fact that the measurement of synergy could easily be conducted in the laboratory led in their opinion "... to a common phenomenon: the study of what can be determined easily, rather than what is necessary [48]." In a recent review of antimicrobial synergy testing, Doern [51] discussed the four primary methods used to assess synergy and data for testing of cystic fibrosis and enterococcal isolates. In the final analysis, he concluded that the four different routinely used laboratory tests for synergy all produced different results and that there was not enough evidence to endorse synergy testing for routine clinical use [51]. Similarly, a meta-analyses review led to the conclusion that "the interactions observed *in vitro* have not been shown to improve patient-related outcomes [52]." Thus, the warning given to physicians more than fifty years ago to exercise caution before using antibiotic combination therapy is still valid today [49].

Combination therapy developed in the 1960s for the treatment of cancer was the first example clearly demonstrating the therapeutic superiority of a drug combination over conventional monotherapy [53]. The seminal development of anti-cancer combination therapy was guided by the quantification of the load of cancer cells in patients and the observation that drugs exhibiting different mechanisms of action could often be combined with additive or synergistic anti-tumor effects [53]. Importantly, these beneficial effects were achieved without a concomitant increase of drug induced adverse effects. Thus, consideration of the adverse effects of drugs in patients was part of the equation from the beginning, and, additive rather than synergistic action was considered adequate if the cancer cells could be reduced sufficiently without harming the patient. This situation is often referred to as "therapeutic synergy" [20,21]. Preclinical anti-cancer drug development, in contrast, was guided predominantly by the search for synergy (*i.e.* supra-additive effects as deviation from Loewe additivity, Bliss independence or response surface models) in cytotoxic effects and the problem of how to overcome or prevent acquired resistance to cytotoxic drugs [21,54]. Over the last ten years, cancer drug development and treatment has begun to shift from non-specific cytotoxic drugs to agents that are targeted at the molecular changes that are driving the development and progression of cancer [54]. The availability of an increasing number of these agents has reinforced the interest in and need for investigations of the effects of drug combinations including high-throughput combinatorial screening for synergy [55].

The paradigm-changing example of targeted cancer therapy is the treatment of chronic myeloid leukemia (CML) with the drug imatinib (Gleevec, STI-571), which achieved long-term survival in most patients. CML is caused by the deregulation of the tyrosine kinase activity of the BCR—ABL fusion oncoprotein, which is the result translocation of between chromosomes 9 and 22. Unrestrained BCR—ABL activity leads to the activation of downstream signaling pathways that stimulate growth and counteract apoptosis. Treatment of CML was revolutionized by the introduction of imatinib, the first BCR—ABL inhibitor. Despite the dramatic success of imatinib and other subsequently developed congeners, however, drug resistant CML (*e.g.* due to the BCR—ABL^{T315I} gatekeeper mutation) and the persistence of minimal residual disease require novel therapeutic approaches [56,57]. In order to address this problem, Winter et al. [57] screened all possible pairwise combinations of 8 BCR—ABL inhibitors for synergistic effects in a cell line expressing imatinib-resistant BCR—ABL^{T315I} and then used a combination of phosphoproteomics, transcriptomics and chemical proteomics to elucidate the molecular basis of the observed drug synergy. Screening was performed using a checkerboard design and synergy was evaluated based on the Bliss independence

model. They observed a pronounced synergy between the pan-aurora kinase inhibitor danusertib and the dual ABL and SRC inhibitor bosutinib. The synergistic effects of both drugs were validated using multifactorial dilutions of both agents and the generation of three-dimensional dose—response surfaces. The ''omics'' work showed that the two drugs exerted their effects *via* ''off-targets'' in the MAPK (mitogen-activated protein kinases) pathway downstream of BCR—ABL resulting in impairment of the transcription factor c-Myc and its target genes [57].

A second even more dramatic example of successful drug combination therapy has been the development in the 1990s of Highly Active Antiretroviral Therapy (HAART) for HIV/AIDS, turning the fatal disease into a chronically manageable although not curable condition [58—60]. The history of the development of HAART and its eventual success was not only a history of scientific advances but ''the result of the passionate 'alliance' toward a common goal between researchers, doctors and nurses, pharmaceutical industries, regulators, public health officials and the community of HIV-infected patients, which is rather unique in the history of medicine [58].'' Scientific advances underpinning the success of HAART were the introduction of non-nucleoside reverse transcriptase (RT) inhibitors and protease inhibitors, the demonstration that viral load was predictive of disease progression and the elucidation of the molecular, functional and clinical impact of drug resistance to antiretroviral drugs (reviewed in Ref. [58]).

Guidelines for combination antiretroviral therapy (cART) of HIV-1 infection recommend daily, life-long treatment with a combination of three drugs consisting of two nucleoside analog RT inhibitors and a non-nucleoside RT inhibitor or a protease inhibitor [61]. Several fixed-dose two or three drug combination pills (once or twice daily) are available and have improved adherence to therapy [61]. Interestingly, ritonavir, which was developed as a protease inhibitor, is a potent inhibitor of cytochrome P-450 3A4 and acts synergistically at low dose (where there is no antiretroviral activity) to increase the activity of better tolerated protease inhibitors [62].

Combinations of drugs are also commonly used in the treatment of rheumatoid arthritis [63,64] and both combination of drugs and fixed dose combination drugs are used in the therapy of cardiovascular disease [65—67]. Efforts to develop combination therapies and combination drugs based on the concept of drug synergy gained significant momentum in the first decade of the 21st century [68—73]. Then and now, the basic idea was to find new uses for existing and clinically established drugs by discovering and exploiting novel, and heretofore unknown synergistic interactions between combinations of these drugs [74].

In 2007, the New York Times published an article under the headline ''Old Drugs In, New Ones Out'' profiling four US biotechnology companies that developed novel combinations of existing drugs [75]. Orexigen Therapeutics, Inc. (San Diego, CA) developed the combination of sustained-release naltrexone and bupropion as weight loss medication to treat obesity. This combination was successfully tested in Phase III clinical trials [76,77]. Celator Pharmaceuticals (headquartered in Princeton, NJ) developed a liposomal formulation of a synergistic 5:1 molar ratio of cytarabine and daunorubicin, two agents commonly used to treat hematologic malignancies, particularly acute myeloid leukemia (AML). POZEN, Inc. (headquartered in Chapel Hill, NC) obtained, in 2008, FDA approval for Treximet®, the combination of sumatriptan and naproxen sodium for the acute treatment of migraine attacks, with or without aura, in adults. In contrast to the focused approach of these two companies, CombinatoRx, Inc. (now Horizon CombinatoRx, Inc. in Cambridge, MA) used what its CEO described as a ''dumb, brute-force, empirical approach'' to screen systematically some two million combinations of 2000 generic drugs in a variety of *in vitro* cellular assays and then selected synergistic combinations in animal models of disease [78,79]. In order to test whether synergism in therapeutic effects might be accompanied by synergy in adverse effects, pairs of drugs were administered in two cell-based *in vitro* assays: a test assay served as a therapeutically relevant disease model, and a control assay modeled a corresponding non-disease state and a ''selectivity index'' was calculated [79]. In an interesting study, CombinatoRx scientists compared data from an *in silico* study with experimental *in vitro* data [80]. In this work, they modeled a branched, unregulated metabolic pathway as a system of Michaelis—Menten enzymatic reactions using ordinary differential equation (ODE) simulation software and then simulated the effect of pairs of inhibiting drugs at varying doses on the activity of the network. They then fitted the data from the numerical simulations to the response surface shape models based on four interaction models: the HSA model, Loewe Additivity model, a modified version of the Bliss Independence model, and a power law based Potentiation model. The data showed that Loewe additive surfaces were produced as expected when both inhibitors targeted the same enzyme. When the inhibitors had separate targets within the pathway, the resulting surfaces were best fit by Bliss boosting, with differing levels depending on the target connectivity.

CombinatoRx scientists then added a second pathway in their simulation connected to the first *via* ''AND'' or ''OR'' type junctions. Branched unregulated pathways produced various levels of Bliss boosting, depending on the target connectivity. When the two pathways were regulated by negative feedback power-law potentiation was observed. Combinations with targets that crossed between the two pathways resulted in more complicated responses including masking effects. In general, strong synergies were observed when the system was inhibited across parallel alternative pathways, but masking effects (buffering) were observed when unrelated pathways were impaired and those pathways were not competing for common precursors. Next, they performed a combination screen focused on sterol metabolism (on which the simulation was based), to compare directly experiment with simulation. Overall, the simulation's accuracy of prediction of the experimental results by the simulation was only 54%. Accuracy reached 72% when only experimentally obtained response surfaces were considered whose best fit was indistinguishable within a certain limit from the predicted shapes.

In a second set of experiments, scientists tested the human colorectal carcinoma cell line HTC 116 using proliferation assays for responses to all pairwise combinations of 90 drugs and probes. Approximately 30% of 4092 drug pairs in the screen produced a formally acceptable fit to any of the

models. For all target connectivity groupings, the optimally sampled combinations exhibited mainly masking and potentiation effects [80].

Similarly, Yin et al. [81] simulated three-node enzymatic networks by ordinary differential equations (ODEs) modeling reactions using Michaelis–Menten kinetics. Simulations were performed with various network topologies and parameters to study the effects of two-drug combinations *in silico*. They simulated drug actions in the network by incorporating drugs as competitive inhibitors into the ODEs and used Loewe Additivity and the CI to detect drug synergy and antagonism. For each drug combination, they computed the EC50-isobole and calculated the CI over the range of concentrations used in the simulation. Drug pairs with CIs between 0.99 and 1.01 were classified as Loewe additive, CI < 0.99 as synergistic and CI > 1.01 as antagonistic. The researchers found that the effects of most of combinations were insensitive to parameter variation, indicating that the effects of drug combinations largely depended on network topology. Synergistic motifs encompassed serial and parallel combinations, while antagonistic combinations were less common and mostly composed of a positive feedback loop and a downstream link.

Cokol et al. [82] examined 200 pairs of antifungal drugs in *Saccharomyces cerevisiae*. They compiled a list of 113 known chemical/target relationships in *S. cerevisiae* from the literature. All compounds known to yield a growth fitness defect by acting as target inhibitors were considered. The resultant drug-target list was integrated with known synergistic genetic interactions to yield a set of 211 drug pairs (33 drugs), predicted to be synergistic according to the parallel pathway inhibition model. Next, the investigators measured the growth rates under all pairwise combinations of seven drug concentrations, linearly increasing from 0 to the minimal inhibitory concentration (MIC). Data were plotted in the form of Loewe Additivity type isobolograms (referred to as ''isophenotypic curves'' by the investigators) and a drug interaction score (α) was computed, quantifying the concavity of the isophenotypic curve ($\alpha = 0$ defined independence, negative or positive values indicated synergistic or antagonistic action, respectively). The scientists generated an error measure by examining the distribution of α for 25 self-self drug combinations. Drug pairs that had significantly smaller or larger α values were classified as synergistic or antagonistic, respectively.

Results showed that 14 of 38 tested drug pairs predicted to be synergistic based on known synergistic genetic interactions exhibited significant synergy (37% accuracy), while 11 of the 38 drug pairs (29%) exhibited significant antagonism. Of the 63 drug pairs that were not predicted to target synergistic gene products, 21 (33%) exhibited synergy nonetheless. Over 90% of the detected synergies were due to only six drugs. The ''promiscuity'' of four drugs was explained assuming that they influenced the bioavailability of the other drugs in the combination *via* specific pathways. Two of the ''promiscuous synergizers'' are known to destabilize the membrane. Interestingly, one drug showed synergistic interactions even in a null mutant of its presumed target gene, suggesting that this drug may interact with additional, yet unknown targets. In fact, after the authors accounted for background rates of synergy for each drug, the results did not show a significant enrichment for drug synergy among drugs thought to target specifically the products of genetically interacting genes.

Torres and colleagues [83] used *S. cerevisiae* for screening all possible pairwise combinations of 11 DNA damaging agents. They found that six combinations were synergistic and three were antagonistic. Interestingly, the strength of two-thirds of these combinations was dependent on the order in which the drugs were added to the cells, and when drugs were added separately with 1-h incubation time between addition of the first and second drug. Testing the synergistic and antagonistic combinations in two human cancer cell lines identified one combination to be synergistic in both cell lines [83]. The investigators converted each dose array to a heat map of inhibitions and degree of synergy or antagonism. The growth curves were normalized to each curve's starting optical density (OD) values and the saturation time point. All OD readings after that time point were set to the saturation OD. Next, the AUC, the area under each growth curve, was calculated. The fractional growth inhibition values for each treatment were calculated by dividing this value by the AUC of vehicle-treated cells. The degree of synergy or antagonism was estimated by the deviation from the Bliss model with the fractional inhibition values.

Tan et al. [84] recently developed a pooled screening method that they named MuSIC (Multiplex Screen for Interacting Compounds) to screen a large collection of 1000 FDA-approved or clinically tested compounds. Rather than screening all pairwise combinations separately, they designed a screening library containing ten compounds in each well of a 384-well plate. This was done to cover all possible pair-wise combinations in less than 3% of the number of wells needed in a pair-wise screen. Pools that contained potentially synergistic interactions were deconvoluted into 45 drug pairs to identify efficacious pairs. Dose titration of the drug pairs was then performed to verify whether drugs acted in synergy. The Bliss Independence and HSA models were used to determine whether or not a drug pair had synergistic effects. When data from selected pairwise combinations were analyzed with both the Bliss Independence model and the CI, findings showed that the CI at the 50% level ''was more lenient'' (CI < 1) than Bliss Independence in diagnosing synergy. The scientists used this method to identify pair-wise drug combinations with anti-HIV activity [84].

Detection of synergy *in vitro* does not guarantee, however, that it will occur *in vivo*. In a recently published study, McCarthy et al. [85] found that the synergy between the cytotoxic anti-cancer drug doxorubicin and the P-glycoprotein inhibitor verapamil observed in cultured murine breast cancer cells (4T1-R) did not improve the overall survival of BALB/c mice with metastatic breast cancer [85].

Investigating how mutations in key DNA repair genes affected the response of *Drosophila melanogaster* to chemotherapeutic agents, Thomas and colleagues [86] discovered that two common polymorphisms in *Drosophila* Cyp6d2 resulted in extreme sensitivity to camptothecin but not its derivatives topotecan or irinotecan. Their findings demonstrate that the genetic background (often not fully characterized) can have a major influence on drug sensitivity and, therefore, skew the results of experiments [86].

Most work concerning the problem of synergy between drugs in combination is limited to drug pairs. Wood et al. [87] investigated if the response of bacteria to combinations of multiple (>2) antibiotic drugs could be predicted by their response to drug pairs. Their starting observation was that

the growth rate of bacteria exposed to pairs of drugs formed by three different drugs could not be predicted from the effects of the single drugs assuming Bliss Independence (*i.e.* some pairs showed pronounced synergy, others pronounced antagonism). Yet, the combination of all three drugs was equal to the product of single drug growth rates suggesting that the drugs acted independently (according to the Bliss Independence model).

Thus, it appears that the strong interactions between the drugs when they were applied in pairs had been eliminated. *Was this due to compensatory interactions?* or *Was this a consequence of simple accumulation of the interaction between drug pairs?* To answer these questions, Wood and colleagues [87] performed parallel experiments and mathematical analysis of the data using a (mechanism independent) statistical approach. Results demonstrated that for a large variety of antimicrobial drug combinations the net effect of the multidrug combination (whether antagonistic, multiplicative, or synergistic compared to the effects of the drug pairs) arose from the cumulative effect of the pairwise interactions and not from new chemical interactions. Most surprisingly, however, was the finding that the bacterial response to combinations of three or four drugs could be calculated using an algebraic expression, which is derived from a famous probability theory theorem (Isserlis' theorem). Wood et al. [87] suggest that that this multidrug ($>$2) response in bacteria may obey statistical, rather than chemical, laws. This idea is consistent with results obtained by Bollenbach and Kishony [88], who investigated how gene expression was regulated in bacteria exposed to pairs of either additive or antagonistic antibiotics. They found that for an additive drug pair, conflicts were resolved by interpolating linearly the single drug responses, while for an antagonistic drug pair, the growth-limiting drug dominated the response. For a given drug pair, the same conflict resolution strategy applied to almost all genes. It would be interesting to know whether or not eukaryotic cells respond to such challenges in a similar way.

Cheok et al. [89] analyzed the expression of over 9600 human genes in leukemia cells from 60 individuals with newly diagnosed acute lymphoblastic leukemia (ALL) before and after *in vivo* treatment with the two cytotoxic drugs methotrexate and mercaptopurine given alone or in combination. Cheok et al. [89] found that 124 genes including those involved in apoptosis, mismatch repair, cell cycle control and stress response discriminated among the four treatments. Only 14% of genes that changed when these medications were given as single agents also changed when they were given together. In a study comparing the transcriptional response of yeast to complex herbal extracts, Cook et al. [90] found that the transcriptional response differentiated between the different extracts.

The concept of synergy is by many considered to be an important factor underpinning the claimed the health promoting effects of fruits and vegetables and therapeutic effects of botanical drugs and their combinations [91—101]. According to Wagner and Ulrich-Merzenich [92], synergy research in herbal pharmacology is aimed at finding "a scientific rational for the therapeutic superiority of many herbal drug extracts derived from traditional medicine as compared with single constituents thereof. "In a second paper by these authors, synergistic effects are "understood as true overadditive effects." [100]

The therapeutic effects of plants are thought to be due to so-called secondary metabolites, a large number of low molecular weight compounds synthesized by plants for signaling and defense. Plants usually produce complex mixtures of secondary metabolites, often belonging to different chemical classes, such as alkaloids, phenolics or terpenoids. It is thought that individual components of a mixture may exert synergistic effects [102]. Stermitz et al. [103] described an example of synergism between two secondary metabolites of the medicinal plant *Berberis fremontii*. They found that in addition to the alkaloid berberine, *Berberis* medicinal plants also synthesized an inhibitor of the NorA multidrug resistance (MDR) pump of a human pathogen *Staphylococcus aureus*. The inhibitor was identified as 5′-methoxyhydnocarpin (5′-MHC), previously reported as a minor component of a traditional therapy for leprosy. The 5′-MHC exhibited no antimicrobial activity alone but strongly potentiated the action of berberine and other NorA substrates against *S. aureus*. Berberine accumulation in the cells was increased strongly in the presence of 5′-MHC, indicating that this plant compound effectively disabled the bacterial resistance mechanism against this antimicrobial natural product [103].

Combination drug therapy is a fundamental principle of traditional Chinese medicine (TCM) [104]. To date, some 200,000 prescription formulas have been recorded. Each component of a prescription has a specific function. The principal ingredient is directed at the main cause and/or symptoms of the disease. A second remedy may also be directed at the underlying cause(s) of the disease as well as accompanying symptoms and complications. Additional components may be added to help the leading ingredients achieve their curative effects by treating any secondary symptoms of the disease and by counteracting any potential adverse effects of the primary drugs. Finally, ingredients may be added that direct the action of all other remedies into the right 'channels' to ensure that together, these medicines do not exceed the patient's capacity to cope with their actions. Thus, in TCM, prescriptions are designed to maximize therapeutic efficacy by facilitating synergistic actions (mutual reinforcement) and ameliorating (mutual restraint) or preventing (mutual detoxification) potential adverse effects while targeting one or several pathophysiological mechanisms (mutual assistance) [104]. Applying these principles, Chinese TCM doctors designed in the1980s the Realgar-*Indigo naturalis* formula (RIF) for the treatment of acute promyelocytic leukemia (APL) [105]. Realgar (As_4S_4) is the principal drug, *Indigo naturalis*, *Salvia miltiorrhiza*, and *Pseudostellaria heterophylla* assist the effects of realgar. *I. naturalis* is qingdai, a traditional Chinese medicine, which is dark blue powder that is prepared from the leaves of *Baphicacanthus cusia*, *Polygonum tinctorium*, *Isatis indigotica*, *Indigofera suffrutticosa* and *Indigofera tinctoria* [106]. In a Phase III clinical trial, a combination treatment consisting of this formula and all-trans retinoic acid (ATRA) was found to be as effective as the standard treatment (intravenous As_2O_3 combined with ATRA) for both induction and maintenance therapies for newly diagnosed APL [107]. Arsenic sulfide (A; realgar, As_4S_4), indirubin (I; from *I. naturalis*) and tanshinone IIA (T; from *S. miltiorrhiza*) are thought to be the main active ingredients of RIF [108—110]. Wang and colleagues investigated the effects of RIF in a mouse model of APL, a human APL cell line (NB4) and primary cultures of APL patient cells. They

compared the single compounds, pairs of compounds and the triple combination. The triple combination proved to be the most effective. Synergy between the three compounds was quantified using the CI in regard to the induction of terminal differentiation of APL cell lines and primary APL cells harvested from patients. The synergistic effect of ATI was explained at the molecular level by the up-regulation of the transmembrane protein aquaglyceroporin 9 (AQP9) by T and I and AQP9 dependent increase in the intracellular concentration of arsenic, effects on key transcription factors of myelopoiesis and degradation of the promyelocytic leukemia-retinoic acid receptor α (PML-RARα).

Li and colleagues [111] investigated the synergism between baicalin, jasminoidin and desoxycholic acid, three ingredients of qingkailing injection in a mouse model of ischemic stroke (temporary Middle Cerebral Artery Occlusion, tMCAO). Qingkailing injection is one of more than 100 Chinese patent medicines approved for the use of stroke in China [112]. Each drug individually was tested as well as all possible double combinations (each drug at half the single dose) and the triple combination (one third of the single dose). Findings revealed that the only baicalin significantly reduced infarct size compared to the control group. Similarly, only the double combinations that contained baicalin were significantly different from the control group. Animals treated with the triple combination, however, exhibited the largest reduction in infarct size and best neurological score. Overall, the triple combination performed better than the average of a large number of single compounds which have been successfully tested in animal models but failed in the clinic (for a detailed review see Ref. [113]). The researchers also performed a DNA microarray and bioinformatics analysis comparing changes in gene expression following drug treatment and interpreted their results in the context of Chinese medicine prescription formula philosophy.

An *in silico* approach to the elucidation of synergistic actions of traditional Chinese medicines termed NIMS (network target-based identification of multicomponent synergy) was developed by Li et al. [114]. In this approach, agents (drugs) are mapped to their target genes in a previously defined disease network (developed using a combination of text mining of the scientific literature and functional genomics data from DNA microarrays); two scores are then calculated for pairs of agents. A Topology Score (TS) is computed for paired agents based on the importance of the target genes in the network and the shortest path between them. A Synergy Score (S) is obtained for each pair of agents by multiplying the TS by an agent similarity score, which is computed from data obtained by text mining [114]. A somewhat similar method but based on interaction networks of proteins known to be changed in a particular disease was developed by Vitali et al. [115]. In their work, they introduced a score they called Topological Score of Drug Synergy (TSDS), which was computed from topological features of the drug targets [115].

Ulrich-Merzenich et al. [116,117] combined experiment and bioinformatics to compare the effect of a complex herbal extract with a single drug. In the experiments, they treated male rats with a complex willow bark extract, a willow bark ethanol fraction or the tricyclic anti-depressant drug imipramine and then analyzed gene expression profiles from the peripheral blood to calculate thresholds for theoretical potential adverse events. Findings showed that the total number of significantly upregulated or downregulated genes was similar between the complex willow bark extract (n = 1673) and imipramine (n = 1733) but significantly smaller in the willow bark ethanol fraction treated rats (n = 117). Interestingly, the number of disease clusters associated with potential adverse events was 13 for imipramine (consistent with known adverse events observed with this drug), five for the ethanol fraction and only one for the willow bark extract [116].

3. Synergy in molecular genetics and development

Progress in eukaryotic molecular genetics has been underpinned by the use of relatively simple model organisms, especially Baker's or Brewer's yeast *S. cerevisiae* (a unicellular fungus), the fruit fly *D. melanogaster*, the roundworm *Caenorhabditis elegans*, the zebrafish *Danio rerio* and the mustard weed *Arabidopsis thaliana* [118—122]. These model organisms are all small, inexpensive to maintain, easy to grow and to manipulate in the laboratory. They have a short reproductive cycle, can be mutated easily, and strains can be frozen and stored for the long term and shared between investigators. *S. cerevisiae* has ~6000 genes, *Drosophila* ~15,000, and *C. elegans* has ~20,000 genes. Hundreds of known human disease genes have homologues in these model organisms, and human genes can be expressed and studied in yeast and flies (see below).

S. cerevisiae was at the very first eukaryotic organism that had its genome sequenced in its entirety [123,124]. Most recently, the native chromosome III of *S. cerevisiae* was replaced by *synIII*, the first eukaryotic chromosome to be entirely designed and synthesized in the laboratory paving the way for eukaryotic "designer" genomes [125]. Over twenty years ago, yeast was used to develop the first system to identify and characterize systematically protein—protein interactions [126,127]. Expression of foreign proteins and peptides on the surface of yeast cells (molecular surface display) has been developed for screening and engineering applications [128,129].

Virtually every "omics" approach was first developed in yeast [130] including recent techniques aimed at characterizing the three-dimensional architectures of genomes [131]. A map of the quantitative interactions of ~75% of all yeast genes was constructed by examining 5.4 million gene—gene pairs for synthetic genetic interactions [132]. In addition, scientists have generated a collection of molecular-barcoded homozygous and heterozygous diploid strains with deletions of each of 5916 genes (including 1159 essential genes) and one haploid strain of each mating type for 4757 non-essential genes [133]. The library of heterozygous diploid strains has been used for chemogenomic profiling [134,135]. Genome-wide functional interactions of chemical compounds can be assayed by their effects on the growth of molecularly barcoded heterozygous *S. cerevisiae* deletion strains that are grown together in a single culture [136]. DNA is prepared from the cultured cells and the molecular barcodes are amplified by polymerase chain reaction using labeled primers. The amplified barcodes are finally hybridized to oligonucleotide microarrays [137]. Jansen et al. [138] developed a bioinformatics approach that efficiently

predicted compound synergy for combinatorial therapies from such chemogenomic profiles and validated their predictions experimentally in *S. cerevisiae* and *Candida albicans*.

Drug resistant yeast mutants can be complemented with a library of barcoded Open Reading Frames (ORFs) of wild type genes [139]. When the transformants are grown in parallel with the presence or absence of the drug, resistant yeast containing the wild type ORF will become drug sensitive and be inhibited in the presence of the drug. DNA from the transformants can be assayed with barcode microarrays. The gene(s) underlying drug resistance can be identified because DNA from wild type complemented transformants will be depleted and the signal on the microarray reduced.

Yeast mutants with growth defects can also be complemented by expression of human genes to deduce and study their function [128].

Functional relationships between genes have traditionally been investigated by generating double mutants and performing so-called synthetic lethal screens [118]. Geneticists use the terms synthetic lethality when the interaction of two mutations is lethal in double mutants but single mutants are viable [140,141]. Perez-Perez et al. [141] refer to synthetic lethal or synthetic enhancement in double mutants as synergistic interactions. According to these authors, a double mutant phenotype can be considered: (1) additive, when it exhibits a combination of traits present in the single mutants; (2) epistatic, when it resembles the phenotype of one of the single mutants, but not the other; (3) suppressed, when it is closer to the wild-type condition than either of the single mutants; or (4) synergistic (also called super-additive or more-than-additive), when the joint contribution to the phenotype made by both mutations is greater than the sum of their individual effects. While additivity is often viewed as a sign of absence of a functional relationship between the mutated genes, epistasis and suppression are thought to indicate an interaction, the interpretation of synergy is controversial because it is not always easy to distinguish it from additivity as the criteria for doing so are often subjective [141]. The term epistasis is used in the field of systems biology more generally when the phenotype caused by multiple mutations or perturbations of any kind (*e.g.* by exposure of an organism to combination of drugs) is different from what is expected based on the individual ones [137,142,143]. An information theoretic approach aimed at detecting interactions of multiple genes within complex pathways in gene expression data has been developed by Anastassiou [114]. They developed a method to discover the cooperative rather than independent contributions of synergistically interacting genes to a phenotype.

Large-scale, often genome wide genetic interaction screens in yeast and other model systems as well as modeling of gene interaction networks *in silico* indicate that genetic interactions and their network topology appear to be conserved between evolutionarily distantly related organisms [132,143—145]. An interesting example was reported by Louie et al. [146] who used yeast to study the genetic interaction network of an analog of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR-ΔF508), the single allele that accounts for most of the disease. They found that yeast gene interactions influencing Yor1-ΔF (the yeast homolog of CFTR-ΔF) biogenesis were representative of human homologs previously found to modulate processing of CFTR-ΔF in mammalian cells. Pallavi and colleagues [147] investigated synergy between genes (notch and mef2) influencing proliferation and metastasis and their downstream signaling pathways. As both genes are co-expressed in breast cancer, these findings have implications for human cancer development and treatment.

C. elegans has become a popular model for studies of aging. The synergistic effect on the life span of *C. elegans* of double mutation of two genes (daf-2 and rsks-1) that code for key molecules in the insulin/IGF-1 signaling and Target of Rapamycin (TOR) pathways were investigated by Chen and colleagues [148].

To a large degree, the molecular basis of development has been elucidated first in *Drosophila*. Development of the *Drosophila* embryo is guided by transcription factors that diffuse through the early syncytium to form morphogenetic gradients. For example, these gradients determine the anteroposterior and dorsoventral axes and the terminal regions of the developing embryo. Simpson-Brose and colleagues [149] found that two morphogens named bicoid (provided by the mother to the egg) and hunchback (both provided by the mother and produced by the zygote) act synergistically to activate a number of other transcription factors to form the correct polarity of the embryo. Twenty years after this report, Lucas and colleagues [150] have reported live imaging of the changes in bicoid dependent hunchback transcription in *Drosophila* embryos. Vertebrate homologues of genes with synergistic effects in *Drosophila* eye development have been found to act synergistically in vertebrate muscle development [151,152].

Zebrafish are an amenable model of vertebrate development. Developing zebrafish embryos are transparent and many mutant lines are available. Hans et al. [153] studied the role of the transcription factors pax8 and pax2a in ear development and found that they acted synergistically, *i.e.* in a collaborative manner, in ear specification.

4. Synergy in biochemistry

The taste receptors of the human tongue belong to the family of G-Protein Coupled Receptors (GPCRs). Many GPCRs are known to be subject to allosteric modulation. So-called Positive Allosteric Modulators (PAMs) enhance the activity of the receptors but have no activity of their own, *i.e.* the combination of an agonist and a PAM exhibits synergy [154]. Scientists of the biotech company Senomyx, Inc., and The Coca Cola Company, performed an *in vitro* screen for novel PAMs of the human sweet taste receptor. They identified PAMs that are not sweet on their own but synergistically increase the sweet taste associated with sucrose and sucralose (an artificial sweetener), both *in vitro* and in human taste tests [155].

Cellulose is polysaccharide that constitutes a major component of cells walls of green plants as well as wood (40—50%); it is the most abundant biopolymer on earth. Cellulose holds promise as a renewable resource for sustainable bioproduction of fuels and chemicals but its conversion into soluble sugars is currently inefficient and not cost effective [156]. It has long been known that enzymatic degradation of cellulose requires the synergistic action of three types of enzymes (an exo-glucanase, an endo-glucanase and a glucosidase) that hydrolyze cellulose into glucose [157]. In a fascinating study, Ganner et al. [158] used Atomic Force

Microscopy (AFM) to observe these three enzymes at work and elucidate the basis of their synergistic action. They found that the synergism among cellulases was dependent on the morphological features of the cellulose substrate and governed by the co-operativity between enzymes that degraded amorphous regions and those that targeted primarily crystalline regions of the cellulose substrate. Surprisingly, the surface-disrupting activity of the cellulose enzymes depended on the structural features of the substrate, along with the size and packing of crystalline fibers, were key determinants of the overall efficiency of enzymatic cellulose degradation [158].

5. Synergy in environmental toxicology

Two other fields of research in the life sciences where the concept of synergy has traditionally been considered are environmental toxicology and ecology [159]. In 1996, it was reported that combinations of certain pesticides acting as so-called endocrine disruptors were up to 1600 times more potent than the single agents alone, causing a sensation and prompting the U.S. Environmental Protection Agency (EPA) to revise its regulations; however, it was later withdrawn [160,161]. In a systematic review of mixture toxicity studies in environmental toxicology, Cedergreen [162] defined synergy on the basis of Concentration Addition (CA) as a reference model and a threshold of a minimum twofold difference between observed and predicted effect using and including both lethal and sub-lethal endpoints. Cedergreen [162] found that synergy occurred in 7%, 3% and 26% of the 194, 21 and 136 binary pesticide, metal and antifoulant mixtures, respectively. The difference between observed and predicted effect concentrations was rarely more than 10-fold. Cedergreen concluded, ''that true synergistic interactions between chemicals are rare and often occur at high concentrations. Addressing the cumulative rather than synergistic effect of co-occurring chemicals, using standard models such as CA, is therefore regarded as the most important step in the risk assessment of chemical cocktails.''

6. Synergy in ecology

Palmer et al. [163] reported an interesting example of synergy in ecology. These investigators set out to determine how multiple partnerships between a long lived and multiple short lived species might interactively affect lifetime fitness of the long lived species in order to understand the evolution and maintenance of cooperation. They observed how sequential association with four symbiotic ant species affected the long-term fitness of the tropical tree *Acacia drepanolobium*. In monitoring growth and survival of 1750 trees over an eight-year period, they found that tree fitness was enhanced by partnering sequentially with sets of different ant symbionts during tree development even though sets of ants included a ''sterilization parasite'' that prevented reproduction and another that reduced tree survivorship. Trees associated with partner sets that included these ''parasites'' enhanced their lifetime fitness by trading off survivorship and fecundity at different life stages.

Brittain et al. [164] observed the foraging behavior and pollination effectiveness of honey bees in California almond orchards with simple (honey bee only) and diverse (non-Apis bees present) bee communities. They found that in the presence of non-Apis bees, the foraging behavior of honeybees changed and the pollination effectiveness of a single honeybee visit was greater than in orchards where non-Apis bees were absent. This change translated to a greater proportion of fruit set in these orchards. The authors conclude that ''the synergistic combination of *Apis mellifera* and non-Apis bees represents a sustainable way to improve crop pollination services'' and that their ''findings highlight a largely unexplored facilitative component of the benefit of biodiversity to ecosystem services [164].''

How the metabolic networks of organism living in an ecosystem may mutually benefit from each other by sharing their metabolic resources was investigated *in silico* by Christian et al. [165]. They quantified the overall positive synergistic effects resulting from sharing metabolic resources by pairs of organisms and also measured the asymmetry of the interaction. Interestingly, they found that in 98.5% both partners of a pair benefited from the interaction [165].

7. Synergy in synthetic biology and metabolic engineering

Metabolic engineering aims to maximize the production of desired chemical and pharmaceutical products under selected operating conditions in microorganisms (*e.g.* bacteria such as *Escherichia coli*). Metabolic engineers mathematically model intracellular metabolic networks to calculate product yields and devise strategies to design, construct and optimize naturally occurring and artificial intracellular chemical networks through genetic engineering (the selective mutation, deletion, and/or introduction of genes) of the production organism [166]. Synthetic biology is an emerging discipline engaged in fundamental biological research that make use of methods and strategies from engineering in order to design and construct biological circuits and devices (''synthetic cells'') through standardized parts (''biobricks'') [166,167]. How the idea of synergy can inform the practice of synthetic biology and metabolic engineering has been discussed recently by Trosset and Carbonell [167] and practically demonstrated by Shen and Liao [168]. Shen and Liao identified synergy between two metabolic pathways for propanol production in *E. coli*. They performed *in silico* maximum yield calculations of a native pathway and a heterologous pathway and verified experimentally the synergistic effects between the pathways predicted by their model [168]. The increase due to the interaction between the two pathways ranged between 30 and 50% compared to operation of a single pathway. Thus, the synergy definition in this work is similar to the HAS in pharmacology and demonstrates that benefits accrue from interaction even if the effect is less than additive and would be classified as antagonistic rather than synergistic by some popular synergy models.

8. Discussion

The research examples that I have reviewed show that synergy exhibits scale invariance. Small molecules act synergistically in the activation of single receptor molecules. Proteins function synergistically in development, metabolism

and signaling. Synergy was found in the interaction of entire communities of organisms. Synergy manifests itself quantitatively or qualitatively: synergistic effects can be smaller or larger or they can be entirely different from what was expected. Corning [169] broadly defined synergy "... as combined or cooperative effects-literally, the effects produced by things that operate together (parts, elements, or individuals). "This definition of synergy covers the broad spectrum of research that I have reviewed in this paper. It is clear that there is no single mathematical model that can be used uniformly to detect and quantify synergy. Nonetheless, we usually recognize synergy intuitively. Anastassiou [114], for example, speaks of "the intuitive concept that synergy is the additional amount of contribution for a particular task provided by an integrated 'whole' compared with what can best be achieved after breaking the whole into 'parts' by the sum of the contributions of these parts." I believe that the idea of synergy crystallized from the ancient and seminal human experience that more could be achieved by working together rather than alone. In the preface of his book "Natural History of Human Thinking" [170], Tomasello writes "In order to survive and thrive, humans were forced, twice, to find new ways to coordinate their behavior with others in collaborative (and then cultural) activities and to coordinate their intentional states with others in cooperative (and then conventional) communication. And this transformed, twice, the way that humans think." Corning [169] has argued that "synergy of various kinds has played a significant creative role in evolution; it has been a prodigious source of evolutionary novelty. It has been proposed that the functional (selective) advantages associated with various forms of synergistic phenomena have been an important factor in the progressive evolution of complex systems over time." In regard to human evolution, Corning says, "The real key to human evolution, accordingly, was not any single prime mover but the entire suite of cooperative behavioral, cultural, and morphological inventions-a synergy of synergies."

The search for synergy is motivated by the potential benefits it can provide for human health, wellbeing and economy. Most often mentioned in this context is the advent of drug combination therapy. However, *in vitro* synergy does often not translate into clinical benefits. History shows that random, brute force synergy screens have so far not resulted in novel combination drugs. Synergy between enzymes and metabolic pathways holds great potential in metabolic engineering and synthetic biology.

The idea of synergy provides furthermore explanatory and heuristic value in the quest to understand the function of complex biological systems. Kelso regards synergy as "natures way to handle complexity in biological systems" [171]. He defines synergy "as a functional grouping of structural elements (molecules, genes, neurons, muscles, *etc.*) which, together with their supporting metabolic networks, are temporarily constrained to act as a single coherent unit." According to Kelso, "synergies are the elementary *functional* units of living things", just as "atoms and their nuclear components are the elementary constituents of matter." Kelso also pinpoints the origins of synergies, which he regards as "the unique expression of two mechanisms heretofore conceived as independent: self-organization and natural selection." In short, "A synergy is a naturally selected chunk of self-organized behavior."

How synergies arise *de novo* and how they are selected and inherited may be testable using a strategy developed by Erez Braun and colleagues. In a fascinating series of experiments, Braun and colleagues rewired the genome of *S. cerevisiae* in different ways and studied how the cells adapted to the unforeseen challenge [172—178]. They observed that a large fraction of the rewired yeast adapted to the challenge. The adaptation of the yeast was not a consequence of the selection of a rare preexisting phenotype but due to the switch of many cells to an adapted state, which itself was stably inherited over many generations [172,174]. Most interestingly, the rapid adaptation was accompanied by a non-specific and irreproducible genome-wide transcriptional response [173,174]. Although the investigators also detected multiple mutations in the rewired genomes, the cells were able to respond to the challenge by changing the transcriptional response with or without the mutations. David et al. concluded, "This study, therefore, stresses network plasticity as an important property for regulatory adaptation and evolution" [175]. I conjecture that this network plasticity results in the creation of novel synergies between previously non-interacting components of the network. If so, it will be interesting to investigate whether or not the novel synergies are as random as the observed transcriptional response and if so whether the ability to find an adaptive solution in apparent randomness has itself a genetic basis, *i.e.* an evolutionary origin, or if it is merely a consequence of an inherent tendency to self-organize of systems that take energy and order from its environment [179].

9. Conclusions

The idea of synergy has directly driven or informed a large amount of research in the life sciences. The research shows that synergy is a ubiquitous phenomenon at all levels of investigation from the molecular level to ecology. Synergy manifests itself quantitatively or qualitatively: synergistic effects can be smaller or larger or they can be entirely different from what was expected. There is no single mathematical model that can be uniformly used to detect and quantify synergy. Synergy provides benefits for human health, wellbeing and economy. Synergy has explanatory and heuristic value in our quest to understand the function of and in designing complex biological systems. Experimental model systems may make it possible to investigate how synergies arise *de novo* from apparent randomness and to delineate the evolutionary role of the tendency to self-organize of systems that take energy and order from its environment.

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