Cognitive Vitality Reports® are reports written by neuroscientists at the Alzheimer's Drug Discovery Foundation (ADDF). These scientific reports include analysis of drugs, drugs-in-development, drug targets, supplements, nutraceuticals, food/drink, non-pharmacologic interventions, and risk factors. Neuroscientists evaluate the potential benefit (or harm) for brain health, as well as for age-related health concerns that can affect brain health (e.g., cardiovascular diseases, cancers, diabetes/metabolic syndrome). In addition, these reports include evaluation of safety data, from clinical trials if available, and from preclinical models.

A3R Agonists

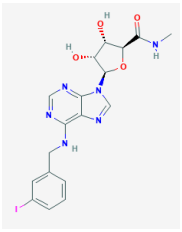
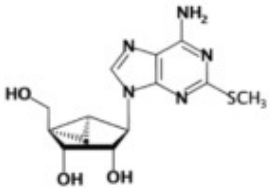
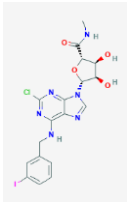
Evidence Summary

Protects tissues against hypoxia and other metabolic stressors. Likely offers clinical benefits for inflammatory and fibrotic diseases and cancer. Clinically tested agonists have a good safety profile.

Neuroprotective Benefit: BBB penetrant A3R agonists may protect against metabolic stress-related neurodegeneration by mitigating inflammation and excitotoxicity, and restoring neuroprotective glial functions.

Aging and related health concerns: Clinical studies show minor benefits in diseases characterized by inflammation and fibrosis. Preclinical studies show cytoprotection during ischemia and mitigation of neuropathic pain.

Safety: Clinically tested A3R agonists are well-tolerated and have a good safety profile. Very high doses may elevate heart rate.

Availability: In clinical trials	**Dose**: Not established	CF101 (IB-MECA) **Chemical formula:** $C_{18}H_{19}IN_6O_4$ **MW**: 510.3 g/mol  Source: PubChem AST-004 Chemical formula: $C_{13}H_{17}N_5O_3S$ MW: 323 g/mol  Source: Liston et al., 2020	CF102 (2-Cl-IB-MECA) **Chemical formula:** $C_{18}H_{18}ClIN_6O_4$ **MW:** 544.7 g/mol  Source: PubChem
Half-life: CF101 $T_{1/2}$= 9 hours CF102 $T_{1/2}$=12 hours FM101 $T_{1/2}$= 18 hours	**BBB**: Varies CF101/CF102 projected to have no/minimal penetrance AST-004 is penetrant		
Clinical trials: CF101: Rheumatoid arthritis (Phase 2 n=79, 253, 254; Phase 3 n=525); Plaque-psoriasis (Phase 2 n=76; Phase 2/3 n=293; Phase 3 n=407); Dry eye (Phase 2 n=80; Phase 3 n=236); Glaucoma (Phase 2 n=89) CF102: Hepatocellular carcinoma (Phase 1/2 n=19, 32; Phase 2 n=78); NAFLD/NASH (Phase 2 n=60) FM101: Phase 1 (n=50)	**Observational studies**: None		

What is it?

Adenosine is an endogenous purine nucleoside that can modulate a variety of physiological processes. The cellular response to adenosine is dependent on the expression pattern of the adenosine receptor subtypes in a given tissue [1]. The adenosine receptors include A1, A2A, A2B, and A3. The A3 receptor (A3R) is the least well characterized subtype, as it plays a limited role in normal physiological function. The adenosine receptors are G-protein coupled receptors (GPCRs). A3R preferentially couples to Gi to inhibit adenyl cyclase leading to a decrease in cyclic adenosine monophosphate (cAMP) levels, and an inhibition of cAMP-dependent signaling kinases/signaling. However, in some cell types and conditions A3R couples to Gq to stimulate the kinase phospholipase C (PLC), and increase levels of intracellular Ca^{2+}. Consequently, the effects of A3R activation are cell-type and context dependent. The expression

pattern and dynamics of A3R is species dependent, thus any studies assessing receptor function in animal models need to be confirmed in human tissue.

A3R is widely expressed, but at a low level under basal conditions, and has a much lower affinity for adenosine than the other receptor subtypes. The concentration of adenosine in the interstitial fluid is typically in the range of 30-300 nM, and in contrast to the A1 and A2 receptors with low nanomolar range affinity, A3R typically has micromolar range affinity for adenosine [2]. N6-methyladenosine (m6A) is released under conditions of cellular stress, and has been identified as another endogenous ligand for A3R [3]. Therefore, **A3R function is most relevant under conditions that stimulate adenosine release, such as metabolic stress** [1]. A variety of A3R agonists have been developed with nanomolar affinity to the receptor. These agonists tend to exhibit bell-shaped dose response curves because at high doses they can activate multiple adenosine receptors, which may have counteracting effects. Several A3R agonists have been developed, however, only two have been tested in clinical trials thus far. A3R agonists are in clinical development for inflammatory autoimmune diseases (rheumatoid arthritis and plaque psoriasis), glaucoma, hepatocellular carcinoma with liver cirrhosis, and non-alcoholic fatty liver disease [4].

The two clinically tested A3R agonists have been developed by the Israeli biopharmaceutical company, Can-Fite Biopharma.
CF101, which is also called Piclidenoson, is an orally bioavailable formulation of the A3R agonist IB-MECA, which shows approximately 50X selectivity for A3R (Ki=1.1 nM) relative to the A1 and A2 receptors. It has been tested in clinical trials for dry eye, glaucoma, rheumatoid arthritis, and plaque-psoriasis. A Phase 3 RCT is currently underway for plaque-psoriasis (NCT03168256).
CF102, which is also called Namodenoson, is an orally bioavailable formulation of the A3R agonist 2-Cl-IB-MECA, which has approximately 2500X and 1400X selectivity for A3R (Ki=0.33nM) relative to the A1 and A2A receptors, respectively. It is being tested in clinical trials for hepatocellular carcinoma with underlying liver cirrhosis (NCT05201404), and non-alcoholic fatty liver disease/ non-alcoholic steatohepatitis (NCT04697810).

FM101 [(2R,3R,4S)-2-[2-chloro-6-(3-chlorobenzylamino)-9H purine-9-yl]-tetrahydrothiophene-3,4-diol] is a biased A3R agonist (Ki =1.44 nM), which acts as an agonist toward G-protein mediated signaling, but acts as an antagonist toward β-arrestin (IC_{50} 44 nM) mediated signaling [5]. Since β-arrestin mediates A3R receptor desensitization, this high affinity agonist may be less limited with respect to the issue of rapid receptor desensitization exhibited by other high affinity agonists. It is being developed by the

South Korean company, Futuremedicine Co. for glaucoma/ocular hypertension (NCT04585100), and non-alcoholic steatohepatitis (NCT04710524).

AST-004 (MRS4322) was discovered to be the bioactive metabolite of the P2Y1 modulator MRS2365 [6]. It acts as an agonist toward the A1R (Ki= 1.59 µM) and A3R (1.49 µM) receptors, but is biased with preferential activity at A3R, and preclinical studies conducted thus far suggest that it does not induce the cardiovascular side effects that are characteristic of selective A1R modulators. Its affinity for A3R is closer to A3R's affinity for endogenous ligands than the high affinity agonists that have been clinically tested, thus far. As a result, AST-004 is expected to modulate A3R (and A1R) in a manner more in line with its endogenous functions. AST-004 is blood brain barrier (BBB) penetrant, with a plasma/cerebrospinal fluid (CSF) ratio of ≈10 in primates [7]. AST-004 is being developed by Astrocyte Pharmaceuticals for traumatic brain injury, stroke, and neurodegenerative diseases. They are planning a Phase 1 study followed by a Phase 2 study in patients with traumatic brain injury (Press Release).

Neuroprotective Benefit: BBB penetrant A3R agonists may protect against metabolic stress-related neurodegeneration by mitigating inflammation and excitotoxicity, and restoring neuroprotective glial functions.

Types of evidence

- Several laboratory studies

Human research to suggest prevention of dementia, prevention of decline, or improved cognitive function? None

Human research to suggest benefits to patients with dementia: None

Mechanisms of action for neuroprotection identified from laboratory and clinical research:
Caution is warranted when interpreting and extrapolating preclinical studies because the tissue profile of expression for A3R varies across species, and the expression is sensitive to environmental conditions [8]. Consequently, the results from *in vitro* cell culture studies are not always consistent with those from *in vivo* animal model studies, and in some cases have been contradictory [9]. This is because the downstream effects of A3R engagement depend on the level of A3R expression, as well as the presence and engagement of other adenosine receptors in a given cell or tissue type [1].

Additionally, the majority of A3R agonists tested thus far are high affinity A3R agonists, with affinities for A3R that are significantly higher than for endogenous ligands (i.e. nanomolar range vs. micromolar range) [10]. These agonists can trigger the desensitization and internalization of the A3R, which may affect the interpretation of results, and account for discrepancies between studies that use acute or chronic treatment. The use of biased A3R agonists may get around the issue of receptor desensitization, and offer a better therapeutic profile for use in chronic conditions.

Due to the pleiotropic effects of A3R activation depending on the physiological conditions and the strength of the agonist, different pathophysiological conditions may preferentially benefit from different biased A3R agonists. In some contexts, modulation of cyclic nucleotide signaling may be preferred, while others may benefit from modulation of calcium-mediated signaling. It will be necessary to characterize the adenosine receptor and signaling landscape of a given condition to determine the optimal A3R modulator.

Alzheimer's disease: POTENTIAL BENEFIT (Preclinical)
Studies from Alzheimer's disease (AD) patients and animal models suggest that adenosine signaling is disrupted in the context of AD. The analysis of hippocampal postmortem tissue indicates a decrease in levels of adenosine receptor A1R and an increase in A2AR, but little is known about A3R in AD patients [11]. A1R is the predominant adenosine receptor subtype in the CNS, however, due to its high expression in the cardiovascular system, it is not considered an optimal therapeutic target for CNS conditions [12]. Although expressed at a far lower level, A3R couples to many of the same G-protein mediated signaling pathways as A1R, including those implicated in mechanisms of neuroprotection. Since A3R expression is low in the cardiovascular system, it offers a more attractive therapeutic option for targeting these pathways without cardiovascular side effects. Consequently, AST-004, which is a BBB penetrant A1R/A3R biased agonist with preferential affinity for A3R, may be particularly suited to CNS conditions [10]. While there is currently no conclusive evidence indicating whether A3R expression or function is altered in the brain in patients with AD, evidence from AD animal models and human data from other conditions suggest that modulation of A3R signaling may be beneficial for AD. It will be necessary to determine whether there is a particular subset of AD patients who would preferentially benefit from A3R modulation, such as those with high brain inflammation or hypometabolism. The restoration of glial function, via metabolic modulation, is one of the proposed mechanisms of action for A3R agonists, at least for AST-004 [10]. Since glial dysfunction has been detected as one of the earliest signs of pathology in the AD brain, this may suggest that A3R agonists may have the greatest utility

during the earliest stages of disease, in reversing detrimental glial changes which contribute to disease progression.
The adenosine receptors can form receptor complexes, and the composition of the complexes can impact the signaling profiles of the receptors [13]. This may be due to the steric hinderance of allosteric sites and/or due to differential affinities of the adenosine receptor subtypes. For example, when in a heteromeric complex with A2AR, which has a much higher affinity for adenosine, A3R-Gi coupling is blocked [13]. In this context, A2AR needs to be inhibited in order to achieve ligand-mediated A3R activation. Consequently, the effects of A3R agonists may depend on the composition of adenosine receptor complexes in a given tissue under particular (patho)physiological conditions. The expression of the A2AR-A3R heteromeric complex was found to be elevated in microglia from $APP^{Swe,Ind}$ AD mice relative to control mice [14]. Furthermore, the signaling downstream of adenosine receptor agonist treatment was altered in AD model microglia. This suggests that the level of these complexes may impact the potential neuroprotective benefit of A3R agonists in a given individual.

The A3R agonist IB-MECA (0.1 mg/kg i.p.) was shown to reduce scopolamine-induced memory impairment on a passive avoidance task in mice when administered immediately after scopolamine [15]. Similarly, the A3R agonist 2-Cl-IB-MECA (200 µg/kg i.p.) protected against streptozotocin-induced memory deficits on object recognition, water maze, and passive avoidance tasks in mice [16]. Conversely, a cell culture study found that use of the A3R agonist 2-Cl-IB-MECA promoted the neuronal internalization of LDL-cholesterol and an associated production of Aβ [17].

A3R agonists have been shown to be neuroprotective with respect to several interrelated aspects of pathophysiology that may be relevant for AD.

Ischemia: The function and regulation of A3R is best understood for its cytoprotective role under the metabolically unfavorable condition of hypoxia (see Aging section) [1]. A3R is normally expressed at a low level in the brain, but expression has been shown to be upregulated in the context of inflammation and hypoxia in numerous preclinical studies [18; 19; 20; 21; 22]. The loss of A3R exacerbates ischemic injury, whereas treatment with A3R agonists is associated with a reduction in inflammation, neuronal loss, and neurological impairment. The protective effect is thought to be mediated by reducing inflammation and excitotoxicity (see below).
One study in the APP23tg AD mouse model found that A3R was upregulated in the hippocampus of pre-symptomatic mice [23]. Since A3R increases under metabolically unfavorable conditions, it suggests that the brain has already adapted to a state of impaired energy metabolism (i.e. oxidative phosphorylation)

in the pre-symptomatic phase, thus this upregulation could be a potential marker of future cognitive decline. When facing a hypoxic challenge these AD mice were unable to further upregulate this neuroprotective program. This adaptation, then, reduces the capacity of the brain to protect itself against additional metabolic/hypoxic insults, leading to an exacerbation of neurodegeneration. However, the use of A3R agonists in other indications, such as arthritis and cancer, has shown that the upregulation of A3R is critical to the therapeutic response of the agonists, suggesting that AD patients with elevated A3R expression may also be more likely to benefit from A3R agonists [4]. In this context, the agonists could potentially restore the neuroprotection associated with elevated A3R signaling.

Inflammation: A3R is overexpressed in the context of inflammation, and has been shown to be involved in both pro- and anti-inflammatory responses, depending on the cell type and environmental conditions [4]. However, in the majority of *in vivo* studies, A3R agonism is associated with anti-inflammatory responses. A3R is expressed in nearly all immune cells, where it mediates the role of adenosine in inflammation. Extracellular adenosine is released through the breakdown of ATP, and increases in the context of metabolic stress, which typically serves to limit cellular damage [1]. Inflammatory cytokines modulate A3R expression in an autocrine manner, such that increased levels of the cytokines leads to an upregulation of A3R, then activation of A3R by its endogenous agonist adenosine limits further inflammation via the inhibition of NF-kB and TNFa, thereby creating a feedback loop [24]. A3R activation also modulates the behavior of immune cells, particularly with respect to chemotaxis and migration [25]. For example, in rats subject to brain ischemic injury, treatment with the A3R agonist LJ529 (1 or 2 mg/kg i.p.) prevented the infiltration of immune cells by inhibiting the microglial chemotaxic response to the chemokine MCP-1 (CCL2) [19].

Excitotoxicity: Adenosine plays a role in regulating neuronal excitability [8]. This is best understood via A1R mediated depression of excitability; however, recent work suggests that A3R may play a similar role in a context-dependent manner. A3R signaling may protect against excitotoxicity-related neuronal loss following metabolic stress. A study in rats suggests that differential expression of A3R may underlie the differential vulnerability to ischemic damage in different regions of the hippocampus [20]. Hippocampal CA1 pyramidal neurons are highly susceptible to damage, whereas CA3 pyramidal neurons are less vulnerable. A3R mediated signaling facilitated neuroprotective synaptic plasticity leading to a decrease in glutamatergic neurotransmission following metabolic stress (ischemia) in CA3 neurons, but this neuroprotective upregulation of A3R did not occur in CA1 neurons. This suggests that vulnerable brain regions may be protected from neurodegeneration by boosting A3R signaling.

Traumatic brain injury: POTENTIAL BENEFIT (Preclinical)
In the controlled cortical impact (CCI) model, mice treated with the A3R agonist, AST-004 (0.22 mg/kg i.p.) 30 minutes following the injury showed less secondary brain injury, including cell loss and BBB breakdown [10]. Treatment improved performance on a fear contextual conditioning task four weeks post-injury. In this model, there were sex differences in post-injury related spatial memory impairment, with only males showing a significant impairment. Treatment prevented this impairment in male mice. Markers associated with glial activation were reduced in the brain following treatment. The proposed mechanism of action for AST-004 involves an increase in energy production in astrocytes, which allows for the restoration of astrocyte-mediated neuroprotective functions, such as ion homeostasis and glutamate recycling. Consistent with this mechanism, AST-004 treatment was found to increase ATP production in astrocytes *in vivo*. This effect is thought to involve A3R activation of the Gq-coupled pathway, resulting in the IP3-mediated release of Ca^{2+} from intracellular stores.
The A3R agonist, MRS5980 (1 mg/kg i.p.) administered one and four hours post CCI also reduced secondary brain injury and neuroinflammation during the acute phase, and preserved performance on memory tasks four weeks post-injury, in this model [26].
These studies suggest that acute activation of A3R shortly after brain injury may mitigate neurological damage. The relevant therapeutic window in humans needs to be established to determine the probability for clinical benefit for patients depending on the post-injury time frame they present for care. Astrocyte Pharmaceuticals has been awarded funding to start Phase 1 clinical testing of AST-004, with plans for a Phase 2 study in patients with traumatic brain injury (Press Release).

APOE4 interactions:
It is not known whether the efficacy for A3R agonists would vary based on ApoE4 status, however, various studies have shown that E4 carriers have a higher degree of metabolic disturbances in the brain, and an associated increase in hyperexcitability even prior to the onset of AD pathology [27]. Since these are conditions that typically trigger the upregulation of A3R and associated cytoprotective responses, it suggests that the endogenous adenosine-A3R system may be dysregulated, such that E4 carriers may preferentially benefit from treatment with A3R agonists.

Aging and related health concerns: Clinical studies show minor benefits in diseases characterized by inflammation and fibrosis. Preclinical studies show cytoprotection during ischemia and mitigation of neuropathic pain.

Types of evidence:

- 10 clinical trials (RCTs for CF101 in rheumatoid arthritis, psoriasis, dry eye, glaucoma; RCTs for CF102 in hepatocellular carcinoma and NAFLD/NASH)
- Numerous laboratory studies

A3R has been found to be overexpressed in a variety of inflammatory autoimmune disorders and cancer. A3R agonists have been shown to have benefits in numerous preclinical models of arthritis, psoriasis, liver cancer, and liver disease [4].

Rheumatoid Arthritis: NO CLINICAL BENEFIT

The A3R agonist CF101 (an oral formulation of IB-MECA also known as Piclidenoson) has been tested in Phase 2 clinical trials for active rheumatoid arthritis, in which it showed efficacy as a monotherapy, but not when used in combination with methotrexate (NCT00280917, NCT00556894) [28]. The discrepancy relates to the dependence on A3R overexpression for clinical efficacy. In a non-placebo-controlled Phase 2 trial (NCT01034306) (n=74) when used as a monotherapy at a dose of 1 mg BID, 55.6%, 33.3%, and 11.5% of the CF101 treated patients achieved at least 20%, 50%, and 70% improvement, respectively, on the American College of Rheumatology (ACR) joint scores [29]. There were significant correlations between the baseline A3R expression level and the ACR50% and ACR70% responses, such that those with the highest baseline levels had the best responses to CF101. Chronic methotrexate use was found to reduce A3R expression levels, which is thought to account for the lack of therapeutic benefit of A3R agonists in this pretreated population [28]. Can-Fite, the biopharmaceutical company developing CF101 has also developed an A3R predictive biomarker kit to identify patients with high A3R who are most likely to respond to the drug (Press Release).

The Phase 3 RCT (NCT02647762) testing CF101 (1 or 2 mg BID) vs. methotrexate in early-stage methotrexate-naïve patients with rheumatoid arthritis (n=252) for up to 24 weeks was terminated early due to the failure to show non-inferiority to methotrexate on the primary endpoint of disease activity score of low disease activity (DAS-LDA) at the interim analysis [30]. The interim results, presented at ACR Convergence 2021, showed that at 12 weeks, disease activity score improvement was seen in 12% at the 1 mg dose and 15% at the 2 mg dose, relative to 24% in the methotrexate group, and 4% in the placebo

group. Based on these results, Can-Fite is no longer pursuing clinical development of CF101 for this indication.

Osteoarthritis: POTENTIAL MINOR BENEFIT (Preclinical)
Synoviocytes are fibroblast-like cells that produce synovial fluid in joints. In the context of joint diseases, such as arthritis, these cells proliferate and take on an altered phenotype involving the production of pro-inflammatory mediators which promote the destruction of cartilage in the joint. A study of synoviocytes from patients with osteoarthritis (n=35) found that adenosine receptors, particularly A2AR and A3R, are expressed and involved in mediating the inflammatory response in these cells by inhibiting p38 MAPK and NF-κB pathways [31]. Treatment of the human synoviocytes with A3R agonists reduced their production of pro-inflammatory cytokines (TNFα, IL-8), suggesting that the agonists may exert protective anti-inflammatory activity in the context of osteoarthritis. A separate study found that the modulation of cytokines (TNF, IL-6, 1L-10) by adenosine in synoviocytes in patients with rheumatoid arthritis or osteoarthritis was primarily mediated by the A2Rs [32], which may explain the lackluster results A3R agonists for arthritis in clinical trials, thus far.
In a rat model of osteoarthritis (monosodium iodoacetate induced), CF101 treatment (100 µg/kg oral 2x/day) starting upon disease onset prevented cartilage damage, osteoclast formation, and bone destruction [33]. CF101 inhibited NF-kB mediated inflammatory signaling, reduced the production of inflammatory cytokines (TNFα), reduced the infiltration of lymphocytes, and promoted the apoptosis of inflammatory immune cells within the joint. In male rats, treatment with CF101 (100 µg/kg 2X/day for five weeks) starting one week after induction of the anterior cruciate ligament-transection model of osteoarthritis, reduced degradation of type II collagen and delayed disease progression [34]. CF101 also reduced the levels of inflammatory cytokines, which was dependent on the enhancement of autophagy in chondrocytes by CF101.
A Phase 2 RCT for CF101 in osteoarthritis was registered (NCT00837291), but subsequently withdrawn by the sponsor. Can-Fite announced that they have been granted a patent for the use of CF101 for the treatment of osteoarthritis in mammals, and appears to be interested in developing it for veterinary use (Press Release).

Cancer: POTENTIAL BENEFIT (Preclinical)
In vitro studies have been mixed as to whether A3R agonists or antagonists are more effective at inhibiting cancer cell proliferation, however, *in vivo* models overwhelmingly support the therapeutic efficacy for A3R agonists in cancer [4]. A3R is highly expressed in solid tumors, and A3R expression on neutrophils and lymphocytes in the blood has been shown to reflect receptor status in the tumors [35].

The hypoxic conditions of the tumor drive the production of adenosine and alters the expression of the adenosine receptors. This process promotes immune suppression and thus fosters tumor growth. In preclinical animal models, A3R agonists have been shown to inhibit tumor growth, inhibit tumor metastasis, and potentiate the anti-cancer effects of chemotherapeutics. The beneficial effects stem from the ability of the A3R agonists to induce different effects on cancer cells and the immune system. In cancer cells, the signaling pathway triggered by A3R activation involves coupling through Gi, which lowers levels of cAMP, and thus inhibits kinase signaling pathways dependent on cAMP such as PKA/PKB/Akt. This ultimately leads to the downregulation of the Wnt/β-catenin signaling pathways that drive tumor growth. Meanwhile, within the context of the cancer environment, A3R agonists activate the ability of the immune system to seek out and destroy cancer cells. Thus far, A3R agonists have only been clinically tested in the context of hepatocellular carcinoma (see below), but preclinical studies suggest they may also be useful for other tumor types characterized by high expression of A3R.
Due to their ability to modulate drug transporters, some preclinical studies suggest that A3R agonists may be useful adjuncts to chemotherapeutics to overcome multidrug resistance. Several A3R ligands have been shown to modulate the multidrug ATP-binding cassette (ABC) transporters, P-glycoprotein (P-gp) and ABCG2 [36]. In a mouse xenograft model (lung cancer cell line A549), the combination of the A3R agonist AB-MECA with the chemotherapeutic doxorubicin had a synergistic effect in reducing lung tumor growth [37]. Similarly, the combination of 2-Cl-IB-MECA with the chemotherapeutics carboplatin and doxorubicin had a synergistic effect in pancreatic (JoPaca-1) and liver (Hep-3B) cancer cell lines [38].

Hepatocellular Carcinoma: POTENTIAL BENEFIT
A3R has been shown to be overexpressed in both tumor tissue and peripheral blood mononuclear cells (PBMCs) (increased by 78% relative to healthy controls) in patients with hepatocellular carcinoma [39]. The expression level of A3R was correlated with levels of NF-kB, a transcription factor associated with pro-inflammatory signaling, which regulates A3R expression as part of a homeostatic feedback loop.
CF102 (an oral formulation of 2-Cl-IB-MECA also known as Namodenoson) has been tested in hepatocellular carcinoma patients in Phase 1/2 (NCT00790218, NCT00790673) and Phase 2 (NCT02128958) clinical trials. In the Phase 1/2 open-label study (NCT00790218) (n=18), mean overall survival of patients treated with CF102 (1, 5, or 25 mg orally BID in 28-day cycles) was 7.8 months [40]. Four patients had disease stabilization and one had regression of tumor metastases. A3R expression was predictive of therapeutic response, as there was a correlation between baseline A3R expression and overall survival following CF102 treatment during this study. A Phase 2 placebo-controlled RCT (n=78) testing CF102 (25 mg BID oral) as a 2nd line therapy in patients with advanced hepatocellular carcinoma and Child-Pugh B (CPB) liver cirrhosis failed to meet its primary endpoint of improved overall survival

(median survival 4.1 months vs placebo 4.3 months) (Hazard Ratio [HR] 0.82, 95% confidence interval [CI] 0.49 to 1.38), but showed a non-statistically significant increase in median survival (6.9 months vs 4.3 months) (HR 0.81, 95% CI 0.45 to 1.43), and a significant increase in the 12 month overall survival rate (44% vs 18%) in the pre-specified subgroup of patients with CPB score 7 (CPB7) [41]. However, there was an imbalance in the study arms, such that all the patients with the highest CPB score (CPB9) were in the CF102 arm, which may have impacted the results. Patients in this study were able to continue treatment with CF102 as part of Israel's compassionate use program (Press Release). According to the sponsor, one of these patients has survived five years and was cleared of all cancer lesions (Press Release). Based on these results, the FDA has approved Can-Fite's Phase 3 study design for CF102 in patients with advanced hepatocellular carcinoma and CPB7 cirrhosis, which is scheduled to begin in Q2 2022 (NCT05201404).

In preclinical models, CF102 has been shown to inhibit tumor cell growth through de-regulation of the PI3K-NF-kB and Wnt/β-catenin signaling pathways. De-regulation of PI3K-NF-kB is associated with the increased expression of pro-apoptotic proteins in cancer cells, while deregulation of Wnt decreased the expression/activity of proteins involved in cell proliferation, such as β-catenin, LEF/TCF, c-myc, and cyclin D [39; 42].

NAFLD/NASH: POTENTIAL BENEFIT

Non-alcoholic steatohepatitis (NASH) is an advanced form of non-alcoholic fatty liver disease (NAFLD), which involves a buildup of fat in the liver leading to inflammatory damage. Liver fibrosis is associated with canonical Wnt/β-catenin signaling.

CF102 (12.5 or 25 mg BID for 12 weeks) has been tested in an exploratory Phase 2a RCT for NAFLD/NASH (n=60) (NCT02927314). There were dose dependent decreases in liver enzymes, serum alanine aminotransferase (ALT) and aspartate transaminase (AST). At week 12, 31.6% in the CF102 group, and 20% in the placebo group showed ALT normalization, though the difference was not statistically significant (P= 0.405) [43]. The proportion of patients with high steatosis scores decreased in the 12.5 mg (from 50% to 31%) and 25 mg (from 43% to 14%) CF102 groups, while it increased in the placebo group (from 33% to 40%). There was also a significant decrease in Fib4-scores (change from baseline −0.08, −0.28, and −0.04 for the 12.5 mg BID, 25 mg BID, and placebo, respectively; P= 0.011 for 25 mg BID vs placebo), suggestive of a potential slowing benefit on fibrosis progression. CF102 (25 mg BID) is currently being tested in a Phase 2b RCT for biopsy-confirmed NASH and F1-3 fibrosis (NCT04697810).

The expression of the A3R gene (ADORA3) was found to be 1.9-fold lower in hepatocytes from patients with NAFLD relative to controls [44]. In other conditions, the efficacy of A3R agonists was dependent on A3R levels. It is unclear if the reduction in hepatic ADORA3 will limit the clinical efficacy of A3R agonists in this population.
In mouse models of NAFLD and NASH, CF102 treatment (100 or 200 µg/kg i.p.) showed anti-inflammatory and anti-steatotic effects [45]. Consistent with the clinical study, treatment normalized ALT levels and improved adiponectin and leptin levels. Anti-inflammatory and anti-fibrotic effects were attributed to the de-regulation of the Wnt/β-catenin pathway in the liver, leading to a reduction in inflammatory NF-kB signaling and α-SMA-associated fibrosis. MRS7476 (5 mg/kg 2X/day orally), which is a water-soluble pro-drug of the A3R agonist MRS5698, improved liver histology, hepatocyte ballooning, and IL-10 production in the STAM mouse model of NASH, but did not improve hepatic fibrosis or inflammation [44].

The biased A3R agonist, FM101 (30 or 60 mg/kg/day), which has agonist activity toward Gi-mediated modulation (inhibition) of adenyl cyclase and cAMP, as well as antagonistic activity toward β-arrestin signaling, was also shown to inhibit steatofibrosis in a high-fat diet induced mouse model of NASH [46]. Treatment mitigated the elevation in liver enzymes and accumulation of fat within the liver, while enhancing mitophagic turnover of damaged mitochondria. FM101 (150 mg and 300 mg BID) is currently being tested in a placebo-controlled Phase 2a RCT in patients with NASH or NAFLD (NCT04710524).

Psoriasis: POTENTIAL MINOR BENEFIT
In a Phase 2 placebo-controlled RCT, patients with plaque-type psoriasis (n=76) were treated with CF101 (1, 2, or 4 mg orally BID) for 12 weeks (NCT00428974). The primary endpoints were safety and change from baseline on the Psoriasis Area and Severity Index (PASI) and physician's global assessment (PGA). There were statistically significant improvements on the mean change on the PASI with CF101 treatment relative to placebo at weeks 8 (P=0.047) and 12 (P=0.031). The 2 mg dose was most effective, with 35.3% of patients (6 out of 17) achieving a PASI ≥50 response [47]. A Phase 2/3 cross-over trial (n=293) testing CF101 (1 or 2 mg orally BID) did not meet its primary endpoint of ≥75% improvement on PASI at week 12, but showed linear improvement over 32 weeks of treatment [48]. A Phase 3 RCT testing CF101 (2 mg or 3 mg orally BID) for 32 weeks in patients with moderate to severe chronic plaque psoriasis (n=528) in comparison to placebo and the active control, apremilast (30mg), will be completed in mid-2022 (NCT03168256). Can-Fite is currently developing a topical formulation of CF101 for psoriasis, which has only been tested in preclinical models, thus far (Press Release).

In the IL-23-induced model of psoriasis, mice treated with the A3R agonists MRS5698 and MRS7344 had reduced immune cell infiltration into the affected area [49].

Neuropathic pain: POTENTIAL BENEFIT (Preclinical)
Adenosine is involved in pain processing in the CNS by regulating neuronal excitability, neural signaling, and glial activation. A3R activation does not alter nociceptive thresholds, thus in preclinical models A3R agonists inhibited pathological but not protective nociceptive responses [50]. A3R agonists have been shown to inhibit mechanical allodynia and hyperalgesia in the context of neuropathic pain. Notably, A3R agonists are projected to lack the abuse potential of opioid-based pain medications because their therapeutic effects are not dependent on the endogenous opioid or endocannabinoid pathways [51]. A3R agonists have been shown to inhibit spinal nociception by decreasing the excitability of dynamic wide range neurons and activating serotoninergic and noradrenergic circuits [51]. In the context of chemotherapy induced peripheral neuropathy, A3R agonists have been shown to modulate spinal neuroinflammatory processes by attenuating astrocyte hyperactivation, reducing the production of pro-inflammatory mediators (TNFα, IL-1β), and increasing the production of anti-inflammatory mediators (IL-10, IL-4) [52; 53]. One study in mice found that the combination of the A3R agonist IB-MECA with a H4 histamine receptor agonist (VUF 8430) was additive in reducing mechano-allodynia in a model of neuropathic pain, due to the enhancement of IL-10 release from T cells [54]. A3R agonists also modulate neurotransmission by inhibiting redox-mediated posttranslational tyrosine nitration and inactivation of glial proteins involved in synaptic glutamate homeostasis [53]. These studies generally involve treatment prior to or concurrent with the therapeutic agent, in which they mitigate the establishment of neuropathic pain. However, a couple of studies found that A3R agonists may also be beneficial in the context of established neuropathic pain [55]. Treatment with the A3R agonist, MRS5980 (1 mg/kg i.p.) during the period of peak pain (eight days after a chronic constriction injury) reduced mechano-allodynia in male and female mice. The effect was dependent on the presence of IL-10 producing T cells, and the presence of a modification on glutamatergic NMDA receptors involved in the regulation of neuronal hypersensitivity. In a separate study, a single dose of IB-MECA (0.5 µmol/kg i.p.) partially reversed hyperalgesia 14 days after a neuropathic pain-inducing injury [56]. Based on preclinical studies suggesting that A3R agonists may augment the anti-cancer activity of chemotherapeutic agents, they may be particularly suited to use as adjuncts to chemotherapy, where they may have both anti-cancer and anti-neuropathy effects [50].

Ischemia: POTENTIAL BENEFIT (Preclinical)

The adenosine-A3R system has been shown to be cytoprotective in the context of ischemic-reperfusion injuries in a variety of organ systems, including the brain, heart, lung, and liver [19; 57; 58; 59]. A3R upregulation is associated with the protective effects of ischemic preconditioning, but it has also been found to exert protective benefits when administered during reperfusion, which is more relevant to clinical application [1]. Activation of A3R in the context of ischemia is associated with a reduction in cell loss within the ischemic tissue as well as reduced infiltration of pro-inflammatory mediator producing immune cell subsets.

Cerebral ischemia: AST-004 was tested in the transient, four-hour middle cerebral artery occlusion stroke model in adult macaques [7]. The non-human primates were treated with an initial bolus followed by a 22-hour infusion of AST-004 starting two hours after the ischemic injury. The dosing regimens ranged from very low (0.11 mg/kg bolus; 0.06 mg/kg per hour infusion) to high (5.2 mg/kg bolus; 2.8 mg/kg per hour infusion). At the mid (1.7 mg/kg bolus; 0.9 mg/kg per hour infusion) and high doses, the slopes of lesion growth were significantly lower than for vehicle-treated animals (composite 48% vs vehicle 71%), and the overall lesion volume (composite) was 30% less than vehicle at 120 hours post-occlusion. The effects on lesion growth rate and volume were correlated with unbound concentrations of AST-004 in the plasma and CSF and to the estimated brain A3R and A1R receptor occupancy levels. There was a non-significant improvement in neurological function, but this study was underpowered for the assessment of neurological outcomes. High A3R interspecies variability reduces the potential translatability of rodent studies, but there is high sequence homology between human and non-human primate A3R, which is expected to increase the translational potential of these findings. Pre-treatment with highly selective A3R agonists, IB-MECA and CI-IB-MECA, has also been shown to be protective against cerebral ischemic injury in rodent models [60; 61]. The protective effect may involve the modulation of glial polarization toward a neuroprotective rather than a pro-inflammatory state. Therapeutic timing has been shown to be critical for efficacy in studies with these potent agonists, however, with the induction of neuroprotection acutely, and the potential exacerbation of neuronal injury with prolonged A3R activation during later stages due to the activation of deleterious calcium signaling cascades [12]. It is unclear whether the receptor desensitization following prolonged activation of A3R by high affinity agonists plays a role in these effects. If so, it may be a drug-specific effect, but if not, it may be a class effect for A3R agonists.

Cardiovascular: POTENTIAL BENEFIT DURING ISCHEMIA (Preclinical)
A3R has low expression on cardiac tissue, but numerous preclinical studies indicate that A3R activation has cardioprotective effects [62], suggesting that its expression and function may be context dependent,

and only contribute to cardiovascular function in a clinically meaningful manner during trauma. The low expression and apparent minimal function in the heart under basal conditions potentially improves the therapeutic profile of A3R agonists relative to A1R and A2R modulators by reducing the risk for cardiovascular side effects [50]. A3R activation is protective in the context of cardiac ischemia by regulating protein kinase C (PKC) activity and mitochondrial K(ATP) channels [62]. Rodent models suggest that A3R does not significantly contribute to atherogenesis or blood pressure regulation, though it is not known whether these findings are species specific or similar in humans. A3R is upregulated on neutrophils in the context of hemorrhagic shock, and the degree of upregulation was found to be correlated with the severity of injury in trauma patients [63]. It is not clear if this is part of a protective adaptive response. In a rodent study, hemorrhagic shock resulted in a decrease in A3R expression on vascular cells, which was associated with a loss of vasoreactivity [64]. Treatment with an A3R agonist (IB-MECA), partially restored vascular reactivity in this model.

Colitis: POTENTIAL BENEFIT (Preclinical)
A3R expression was found to be decreased on epithelial cells in the colon in patients with ulcerative colitis and in a mouse model of DSS-induced colitis [65; 66]. In ulcerative colitis patients (n=18), the decrease in A3R was associated with an upregulation of inflammatory mediators, TNFα, IL-1β, and NF-kB [65]. Treatment of cultured colon tissue from these patients with the A3R agonist 2-Cl-IB-MECA significantly reduced the production of these pro-inflammatory mediators. In the colitis mouse model, 2-Cl-IB-MECA reduced damage to the colonic mucosa and reduced levels of the same set of pro-inflammatory mediators as was seen in the patient tissue [66]. Additionally, A3R agonist treatment reduced the infiltration of neutrophils and levels of myeloperoxidase (MPO) [25; 66].
In a rat model of DNBS-induced colitis, treatment with the novel selective A3R agonist AR170 (1.5 mg/kg i.p.) for six days starting one day prior to induction of colitis, reduced body weight loss and macroscopic damage to the colon, including adhesions and bowel dilations [67]. AR170 also reduced levels of inflammatory mediators (TNF, IL-1β, and MPO) in the colon. Additionally, AR170 treatment reduced visceral hypersensitivity/pain. The A3R agonists MRS5980 and Cl-IB-MECA also reduced visceral pain in the DNBS-induced model of colitis in rats during both the acute and chronic phases [68]. In this model, this effect was mediated via the inhibition of the $Ca_V2.2$ calcium channel.

Ocular disease: MARGINAL POTENTIAL BENEFIT
The preclinical literature has been mixed as to whether A3R agonists or A3R antagonists are more beneficial for ocular diseases, however the discrepancies are likely related to the context dependent nature of adenosine signaling, such that the effects of A3R activation may differ based on the

experimental conditions [1; 69]. Early clinical trials of the A3R agonist CF101 suggested possible benefits for several ocular diseases, but subsequent trials failed to show clinical efficacy.

Glaucoma

Preclinical studies have been mixed as to whether A3R agonists are protective against optic nerve degeneration, but suggest the effects may be cell type dependent. The A3R agonist 2-Cl-IB-MECA exacerbated oligodendrocyte cell death and myelin loss in rat oligodendrocyte cell culture, but protected against retinal ganglion cell death when administered intravitreally in rat models of retinal ischemic injury and optic nerve injury. Increased intraocular pressure (IOP) is one of the major risk factors for glaucoma. It was noted in a Phase 2 RCT for dry eye that treatment with 1 mg CF101 BID for 12 weeks decreased IOP by 1.1 mmHg, which was a significant reduction relative to placebo [70]. However, oral CF101 failed to significantly reduce IOP in a Phase 2 RCT (NCT01033422) in patients with ocular hypertension or glaucoma [4]. Since preclinical and clinical studies used different routes of administration, it is unclear if the pleiotropic effects of systemic A3R agonism impact its efficacy in ocular disease, and whether targeted topical or intravitreal formulations of CF102 would offer higher therapeutic benefit.

Preclinical studies suggest that the biased A3R agonist, FM101, may be a more effective option for lowering IOP [5]. Topical formulations (500 and 750 uM) of FM101 lowered IOP in rabbits and in the DBA/2J glaucoma mouse model [71]. Systemic administration of FM101 (oral 10 or 20 mg/kg) also lowered IOP to a similar extent in a glucocorticoid-induced glaucoma mice model. The reduction to IOP was associated with the remodeling of the trabecular meshwork in a manner to increases outflow of fluid in the eye (aqueous humor).

Dry Eye

CF101 (oral 1mg BID for 12 weeks) significantly improved clinical symptoms of dry eye, including the proportion of patients with more than 25% improvement in corneal staining (CF101 84.6% vs placebo 52.2%), and an improvement in tear meniscus height, in a Phase 2 RCT (n=68) (NCT00349466) [70]. However, the Phase 3 RCT (NCT01235234) testing CF101 for dry eye did not meet its primary endpoint, and clinical development has been discontinued for this indication.

Uveitis

Uveitis is an inflammatory eye condition which can result in blindness. In a mouse model of autoimmune IRPB-induced uveitis, CF101 (10 µg/kg, 2x/day oral) improved pathological manifestations of uveitis such as the infiltration of immune cells, retinal folds and focal retinal detachments [72]. Treatment was

associated with anti-inflammatory effects including, the inhibition of pro-inflammatory cytokines IL-2, TNFα, and IFNg, along with the upregulation of the anti-inflammatory cytokine IL-10. A clinical trial testing CF101 for uveitis was registered (NCT01905124), but was subsequently withdrawn by the sponsor.

Safety: Clinically tested A3R agonists are well-tolerated and have a good safety profile. Very high doses may elevate heart rate.

Types of evidence:

- 12 clinical trials (7 clinical trials for CF101, 4 clinical trials for CF102, 1 clinical trial for FM101)
- Numerous laboratory studies

Since A3R expression is generally low at basal levels, and tends to only increase in the context of trauma, such as metabolic stress, the therapeutic safety profile of A3R modulators is expected to be superior to other modulators of adenosine signaling [4]. Preclinical and clinical safety studies provide further evidence of a good safety profile for A3R agonists.

The A3R agonists CF101 and CF102 have been shown to be well-tolerated and have a good safety profile in patients with rheumatoid arthritis, plaque psoriasis, dry eye, glaucoma, hepatocellular carcinoma, and NAFLD/NASH, as well as in healthy volunteers. The pharmacokinetics were linear, proportional to dose [73]. Can-Fite's annual Drug Safety Update Report for 2020, demonstrates favorable safety profiles for CF101 and CF102, based on clinical testing in over 1,500 patients (Press Release).
In a Phase 1 ascending dose study in healthy men (n=43) for CF101, single oral doses up to 5 mg and multiple doses up to 4 mg were safe and well-tolerated [73]. Single oral doses of 10 mg were associated with flushing, tachycardia, nausea and vomiting, while multiple doses of 5 mg were associated with headache, drowsiness, hot flushes and dizziness. Aside from elevated heart rate at the highest dose, there was no other evidence of adverse cardiac events, based on electrocardiogram monitoring.
In reported Phase 2 and Phase 3 RCTs, the incidence of adverse events was typically similar to placebo. At the 4 mg dose of CF101, three rheumatoid arthritis patients discontinued treatment due to adverse events, including headache/nausea, atrial tachycardia, and an exacerbation of Parkinson's disease [29]. In RCTs testing the 1 to 2 mg dose range, adverse events were mild, and included headache, nausea, and rash [29; 47; 48; 70]. There were two severe adverse events, pruitis and a skin allergic reaction, in patients with psoriasis taking 1 mg BID CF101 [47].

In a Phase 1/2 trial testing CF102 at oral doses up to 25 mg BID in hepatocellular carcinoma, there were no serious drug-related adverse events or dose related toxicities [40]. In a Phase 2 RCT in the same population, treatment related adverse events were primarily grade 1 or 2 for gastrointestinal events or reductions in blood cell counts [41; 74]. One grade 3 adverse event was reported for hyponatremia. In patients with NAFLD/NASH taking CF102 at 12.5 or 25 mg BID, there were three adverse events considered possibly treatment related; myalgia, muscular weakness, and headache [43]. There were no severe treatment-emergent adverse events, no hepatotoxicity, and no deaths in this study.

The biased A3R agonist FM101 was tested in Phase 1 SAD (75 mg to 2400 mg) and MAD (150 mg QD to 450 mg QD and 600 mg BID for one week) studies assessing safety, tolerability, and pharmacokinetics in healthy volunteers (n=50) (NCT03879928). All of the adverse events were mild, with gastrointestinal events, including nausea and diarrhea, as the most common. FM101 was rapidly absorbed with linear pharmacokinetics, and steady-state levels were achieved within one week [75]. It was well-tolerated up to the highest tested doses (2400 mg single dose; 600 mg BID for one week). There were no clinically relevant safety signals on vital signs, laboratory tests, or electrocardiogram parameters.
FM101 has been tested in acute and sub-chronic toxicology studies in rats and dogs [5; 71]. Abnormal posture, irregular respiration, decreased movement, loose stools, and ear flushing were observed during the early phase of dosing in rats that received 500 and 1000 mg/kg/day (orally). No adverse effects were observed related to ophthalmology, clinical chemistry, urine, organ weight, and histopathology [5]. The no evidence of adverse events level (NOAEL) was determined to be 1000 mg/kg/day in rats.

In adult male cynomolgus monkeys, AST-004, administered as an intravenous infusion in the context of a transient middle cerebral artery occlusion ischemic injury, did not exert any clinically relevant changes to physiological parameters during the course of the study [7].

Sources and dosing:

CF101 (Piclidenoson) and CF102 (Namodenoson) are being developed by Can-Fite BioPharma for plaque psoriasis and hepatocellular carcinoma/NASH, respectively, and are currently being tested in RCTs. CF102 is available in Israel for patients with advanced liver cancer as part of a compassionate use program. The derivative compounds IB-MECA (CF101) and 2-Cl-IB-MECA (CF102) are available for research use from commercial suppliers. A therapeutic dose has not yet been established, but CF101 has shown to have a good safety profile at an oral dose of 1 or 2 mg BID, and is currently being tested at oral doses ranging from 1 to 3 mg BID in Phase 3 RCTs. CF102 has been safely tested at oral doses up to 25

mg BID. FM101 is being developed by Futuremedicine Co. for glaucoma and NASH, and has been found to be safe at a single oral dose of 2400 mg and up to 600 mg BID for one week. AST-004 has not yet been clinically tested in humans. It was found to be safe up to a dose of 5.2 mg/kg bolus; 2.8 mg/kg per hour infusion for 22 hours in a stroke model in non-human primates. It is currently formulated for intravenous dosing. Astrocyte Pharmaceuticals is working on developing an oral formulation of AST-004 for use in chronic conditions (Company website).

Research underway:

CF101 is currently being tested in the Phase 3 Comfort™ study for moderate to severe plaque-psoriasis (NCT03168256). Topline results are expected in Q2 2022.
CF101 is currently being tested in patients with Covid-19 (NCT04333472). Due to its anti-inflammatory activity, A3R agonists have been proposed to be good candidates for immunotherapy associated cytokine release syndrome [76].

CF102 is currently being tested in a Phase 2 RCT in patients with NASH and F1-3 fibrosis (NCT04697810). The estimated study completion date is in 2023.
CF102 will be tested in the Phase 3 LIVERATION RCT in patients with advanced hepatocellular Carcinoma with Child-Pugh class B7 cirrhosis (NCT05201404). The estimated completion date is in 2025.

FM101 (150 or 300 mg BID) is being tested in a Phase 1/2a trial in patients with ocular hypertension and to assess the bioavailability of an oral tablet formulation relative to an oral solution in healthy volunteers (NCT04585100). The estimated completion date is in 2023.
FM101 (150 mg or 300 mg BID) is being tested in a Phase 2a trial in patients with NASH or NAFLD (NCT04710524). It is expected to be completed in 2022.

Search terms:
Pubmed, Google: A3R, CF101, CF102, IB-MECA, 2-Cl-IB-MECA, AST-004

- Alzheimer's diease, neurodegeneration, ischemia, inflammation, cancer, arthritis, cardiovascular, neuropathy, glacuoma, clinical trial, safety

Websites visited for A3R agonists:

- Clinicaltrials.gov (CF101, CF102, FM101)
- PubChem (CF101, CF102)

- DrugBank.ca (CF101, CF102)
- Cafepharma (CF101, CF102)

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